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SPECIAL NUMBER DEDICATED TO DR. LEO LOEB

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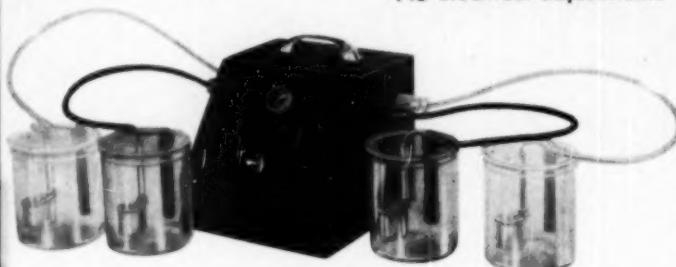
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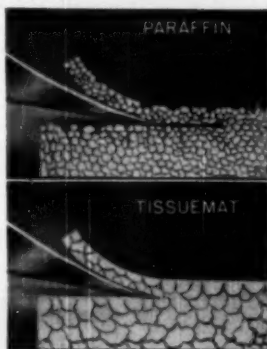
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DR. LEO LOEB SPECIAL NUMBER

This Special Issue of the
A. M. A. Archives of Pathology

is Dedicated to
DR. LEO LOEB

In Recognition of His Outstanding Contributions to Pathology

Committee for the Dr. Leo Loeb Special Number

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FOREWORD

It is proper from time to time to survey medical science and assess the progress which has been made. One way of doing this is to issue special numbers of scientific journals in honor of those individuals who have made significant contributions to this progress; that is, to those who in another connection have been designated as the "giants."

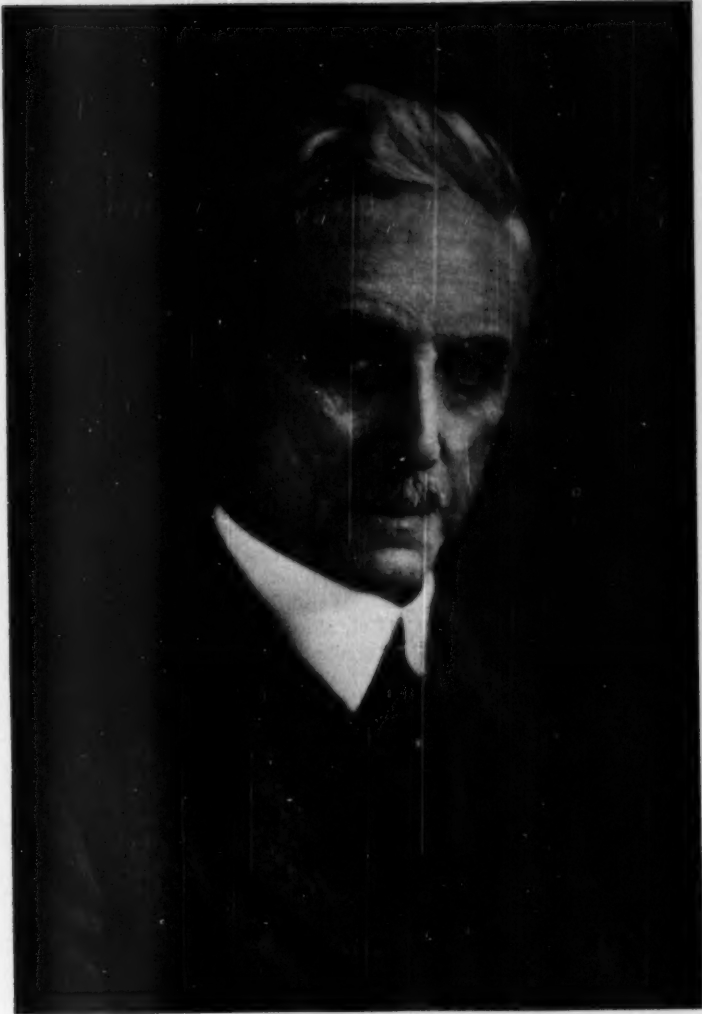
Dr. Leo Loeb has had the privilege of living and working in the time which has been called "The Heroic Age of American Medicine." His scientific work has encompassed not only a period of over fifty years, but a similar broad horizon of problems.

The publications of Dr. Loeb are numerous and varied, as testified to by the list included in this volume. But, perhaps even more important, this volume is proof that his ideas, his standards of excellence, and his manifold interests have been multiplied many times through his students.

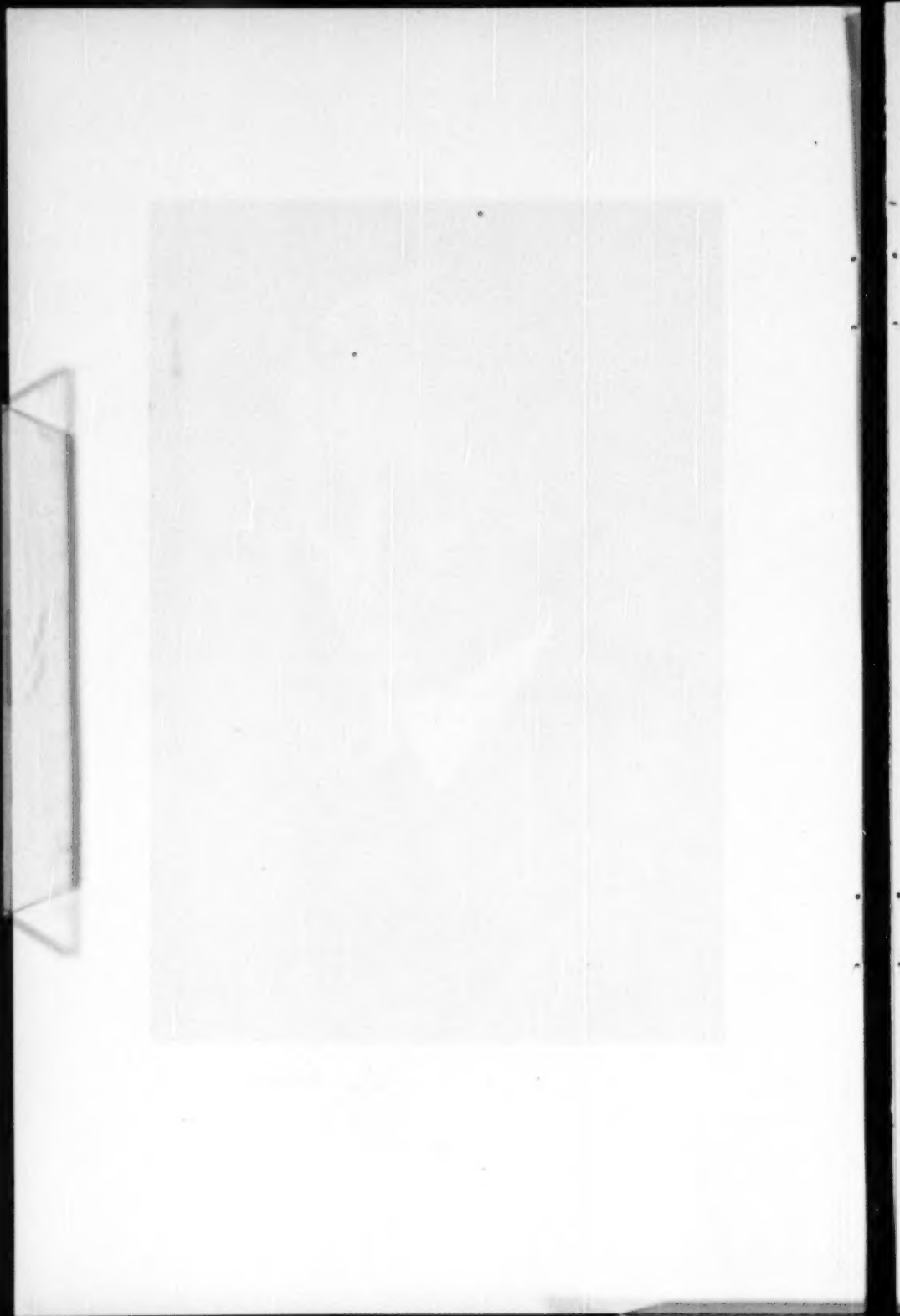
May this volume serve only as a milestone in the continuing contributions and influence of Dr. Loeb and of his students on medicine and pathology.

ROBERT A. MOORE

Edward Mallinckrodt Professor of Pathology
and Dean, Washington University School of
Medicine



Leo Loebl



A. M. A. ARCHIVES OF PATHOLOGY

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BIOGRAPHICAL NOTES ON DR. LEO LOEB

To accompany this *Festschrift* for Dr. Leo Loeb, contributed by a number of his pupils and co-workers in celebration of his eighty-first birthday, the committee in charge asked another of his friends—one not qualified to participate as they do—to write a biographic sketch which might include brief comments of a personal character.

It is a privilege to be thus included with the group of closer colleagues in paying tribute to a notable pioneer in the broad development of experimental pathology during the past half century.

Underlying the long-continued scholarly, distinguished and prolific contributions by Dr. Loeb to experimental biology and medicine, one likes to think of the personal qualities that have inspired and directed his efforts. From a friendship now lasting almost 50 years I can testify to the intellectual vigor and integrity which offset a somewhat frail but resilient physique, and to the keen sensibilities and insight united with a kindly judicial attitude which have guided his opinions and actions. Using his own terminology, these qualities may be called the elements of his personal "physical and organismal differentials" which constitute the basis of his "unique individuality."

Leo Loeb was born on Sept. 21, 1869, in Mayen, Germany. He studied natural sciences and medicine at the universities of Heidelberg, Berlin, Freiburg and Zurich from 1889 to 1896. After receiving the M.D. degree from the last-named university, he came to the United States and went to Chicago, where his brother Jacques Loeb had recently been appointed professor of physiology in the new university. Leo Loeb's first academic position was adjunct professor of pathology at the University of Illinois (Chicago) in 1900, where he had already conducted studies on topics which reveal the direction of his major interests.

His first recorded paper (1896) bears the title "Diagnosis of Tumors of the Supraorbital Arch"; the second, published the following year, described results of "Transplantation of White Skin on Black Skin (and Reverse) in the Ear of Guinea Pigs"; the third and five or six that followed were all concerned with experimental studies of tumor growth. In 1903 he accepted a research fellowship at McGill and a year later became assistant professor of experimental pathology at the University of Pennsylvania. During the following six years more than 80 papers appeared, describing his work on blood coagulation, liver necrosis, edema and especially on cancer researches involving transplantation of tumors, a number of them in collaboration with Elizabeth Cooke, M. S. Fleischer and others. The work of these years established the reputation of

Dr. Loeb in the field of experimental pathology, especially in cancer research.

In 1910 he was selected as the director of laboratories for experimental studies of cancer in the newly created Barnard Free Skin and Cancer Hospital in St. Louis. After holding that post for five years, he became professor of comparative pathology at Washington University in 1915. Following the resignation of E. L. Opie in 1924, Dr. Loeb was appointed professor of pathology and head of that department. In this position his influence spread throughout the institution, especially among numerous students and young associates who were assistants and associates in his research. Since his retirement in 1937 to become emeritus professor he has continued his work in quarters provided by the Oscar Johnson Institute of the same university.

In this sketch no attempt will be made to characterize or assess expertly or in detail the varied contributions Dr. Loeb has made to experimental pathology. But long acquaintance with biochemical aspects of some of the areas which Dr. Loeb has cultivated and working in quarters more or less adjacent to his laboratories give one impressions that may justify a few comments appropriate for this sketch. It is my opinion that Dr. Loeb, more than any other investigator of his time, inaugurated in this country and has continued to forecast the experimental approach to the study of cancer, and that by his work he has laid much of the background on which the widespread interest and activity in that subject now stand. His influence has been exerted by logical continuity and persistence of effort, by the fertility of his ideas and by personal example.

Around the turn of the century, he described on the basis of experimental data characteristics of tumor and tissue growth and shares with Ross Harrison the credit for methods of tissue culture *in vitro*. His continued studies of tissue transplantation, culminating in the monumental volume "The Biological Basis of Individuality" (1945), constitute a major contribution to biology as well as to medical and surgical knowledge. His work on edema, summarized in a monograph (1923), his studies of the sexual cycle, and especially his notable experiments demonstrating the interaction of endocrine glands—thyroid, anterior pituitary and sex glands—are widely recognized as pointing the way to great advances later accomplished by many others in these fields.

Dr. Loeb's colleagues at Washington University gave expression several years ago to their collective estimate of his achievements and indicated their regard for him as a scholar and scientist by proposing him for the highest honor within their power to bestow: to be awarded the degree Doctor of Science by the university (rarely awarded to members of its staffs).

We who know him well wish Leo Loeb continued good health—supported by the satisfaction of having made significant contributions to the advance of knowledge for the benefit of mankind.

PHILIP A. SHAFFER.

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ABNORMALITIES IN THE BEAKS OF DEVELOPING CHICK EMBRYOS

Postmortem Changes Which May Be Confused with Developmental Anomalies

RUSSELL J. BLATTNER, M.D.
AND
ALICE POLK WILLIAMSON, A.B.
HOUSTON, TEXAS

IN THE COURSE of investigations being carried out as part of a study concerned with the effect of virus infection on the development of the chick embryo,¹ striking changes were observed in the gross appearance of the beak structures of a significant number of the embryos studied. At first it seemed reasonable to consider these changes abnormalities of development occurring as a result of virus infection. However, more critical analysis of infected and control embryos revealed that often such embryos were examined for abnormalities after death had taken place. These observations were particularly confusing in the preliminary experiments in which the inoculum of fowlpox virus used was almost invariably lethal for the embryo.¹ It became apparent that the beak changes were found in dead embryos only, and subsequent studies revealed that the "abnormalities" were not caused by the virus infection but in reality were postmortem changes. Figure 1 shows typical examples of the beak changes: shortened lower beak, thickening and protrusion of tissue on each side of the lower beak immediately below the angle of the jaw, moderate to extreme twisting of the beak to one side or the other, crossed or scissored beak, and occasionally a bending down of the upper beak giving a parrot-like appearance.

Since the beak changes noted in these studies were definite and seemed consistent in type, and because such artefact changes might be confused readily with developmental defects, it seemed worth while to ascertain under what circumstances these beak changes can occur, and to establish the nature of the process involved in their production.

METHODS AND MATERIALS

White leghorn eggs incubated in the laboratory at 38 C. were used routinely in all the experimental work. A small number of preliminary experiments

From the Department of Pediatrics, Baylor University College of Medicine.
1. Report in progress.

had been conducted in which eggs from a flock of White Rocks incubated at a local hatchery were used. There were no detectable differences in the beak changes observed in eggs from the two different strains of chickens.

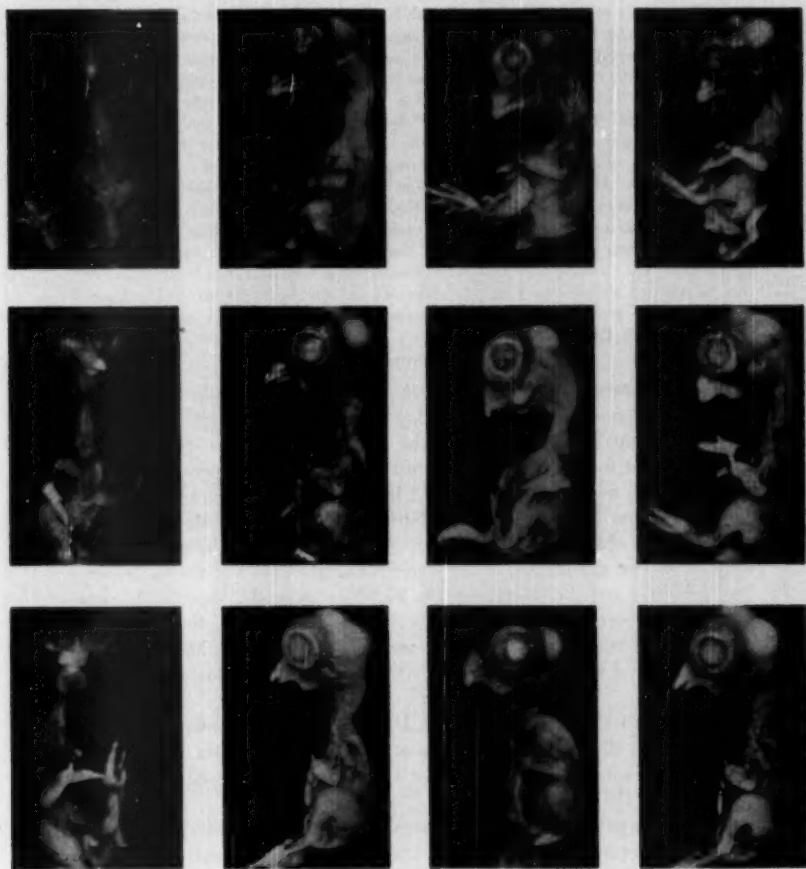


Fig. 1.—Chick embryos, nine days' incubation: Upper row, appearance of normal embryos dead approximately two hours. Middle and lower rows, abnormal appearance of beaks in normal embryos dead approximately 20 hours.

Normal embryos in various stages of development were killed in one of the following ways: by clipping the vitelline artery of the chorioallantoic membrane; by coating the egg shell with paraffin, which interfered with the respiration of the embryo; by decapitating the embryo. The beak changes observed in embryos

killed by these various methods were similar. The paraffin-coating method proved to be the procedure most satisfactory for experimental use. In any instances in which the egg shell was opened, exposing the embryo, aseptic precautions were followed, and broth culture checks for possible contamination were made routinely.

Preliminary experiments revealed that in embryos of eight to 14 days' incubation death takes place from two to three hours after the shell is completely covered with warm melted paraffin. In order to insure a reasonable interval of time after the death of the embryo, a period of four hours after the paraffin-coating operation was adopted routinely for inspection of a number of embryos from each experimental group. No embryo was ever found alive after this interval, and no beak abnormality was ever observed in chicks dead four hours after paraffin-coating of the egg shell. In most instances the eggs were returned to the standard egg incubator kept at 38 C. after the paraffin-coating procedure. In those experiments necessitating windows in the eggs, or those in which the embryos were removed from their shells and transferred to flasks containing fluids, it was more convenient to place the eggs and the flasks in an incubator of bacteriologic type at 38 C. Conditions of moisture were likewise comparable in the two incubators, and no differences could be observed in the incidence of beak changes whether the period following the experimental procedure was passed in one incubator or in the other.

RESULTS

Time Interval Between the Death of the Embryo and the Appearance of Beak Changes.—Forty-two 10 day old embryonated eggs were coated with paraffin and returned to the incubator. Groups of six were examined at four hour intervals throughout a 24 hour period, and one group of six was examined after 48 hours. Of those embryos examined at four hours following the paraffin-coating procedure, all were dead, but no beak changes were observed. After eight hours, slight thickening was apparent at the base of the lower beak. At 12 hours the thickening was definite and the discrepancy in length between the upper and lower beaks had begun to be evident. By 16 hours apparently the maximum change had occurred, since those embryos inspected at 20, 24 and 48 hours showed no greater deviation from normal than those inspected at 16 hours.

Of the 24 embryos examined at 16 hours or later, 23 showed a short lower beak; all showed protrusions at the base of the lower beak; seven showed twisting of the beak to the right, and nine showed twisting of the beak to the left (fig. 2).

This experiment was repeated using 14 embryonated eggs of nine days' incubation, embryos being removed for examination at each interval of time as before (fig. 3). Results were approximately the same as those obtained with 10 day old embryos. Twisting of the beak was found in every case in the 9 day old embryos examined 16 hours or longer after the paraffin coating, and the changes in the lower beak seemed even more pronounced than in the 10 day old group.

The Relation of Age of the Embryo to the Occurrence of Beak Changes.—Since certain embryonic periods may be more critical than

others for the development of given structures, it was of interest to determine whether these abnormal beaks are restricted to specific age groups or whether such beak changes occur to some degree in all dead embryos which have reached the stage of beak development. Accordingly, 48 fertile eggs of known age since laying were placed in the incubator. After eight days' incubation, 12 eggs were removed, coated with paraffin and returned to the incubator. Groups of four were examined at 4, 16 and 24 hours after the paraffin-coating procedure.

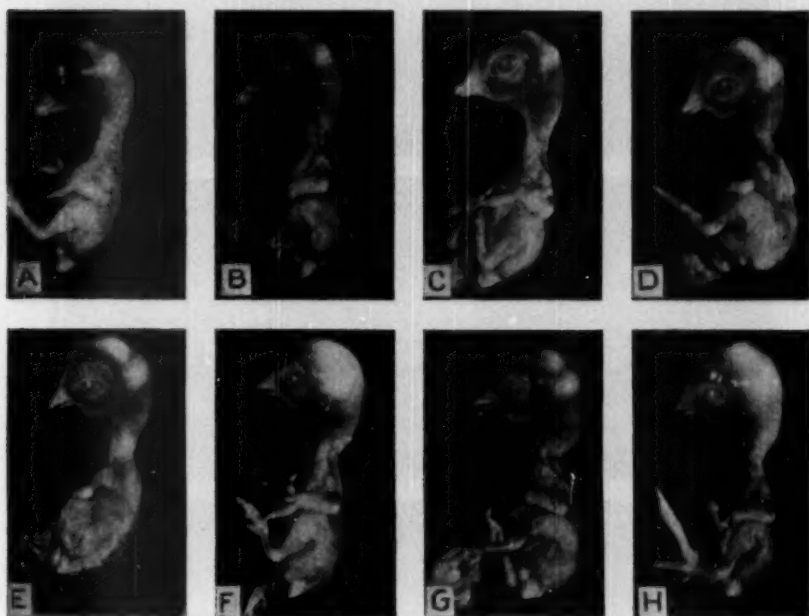


Fig. 2.—Chick embryos, 10 days' incubation, showing progressive changes in the beak after death of the embryo. Time after death: *A*, normal; *B*, 2 hours; *C*, 6 hours; *D*, 10 hours; *E*, 14 hours; *F*, 18 hours; *G*, 22 hours; *H*, 48 hours.

From the original 48 eggs, groups of 12 were subjected to paraffin coating at 10 days', 12 days' and 14 days' incubation respectively. Eight day old embryos did not show any marked beak changes when left in the incubator, 16 to 24 hours after death. However, in the embryos of 10 days' incubation at the time of the procedure, the beak changes were definite and striking. Of embryos with 12 days of incubation at the time of paraffin coating, one or two showed some thickening

at the base of the lower beak, but no striking beak changes in any way comparable to those observed in 10 day embryos were seen. In no case was there twisting of the beak as a whole, or any discrepancy between the lengths of the upper and lower beaks. In embryos subjected to the procedure at 14 days' incubation no changes of this type were found. The photographs in figure 4 show embryos at each age level and illustrate the characteristic results obtained.

These observations seem to indicate that beak changes take place in dead embryos of more than eight days' but less than 12 days'

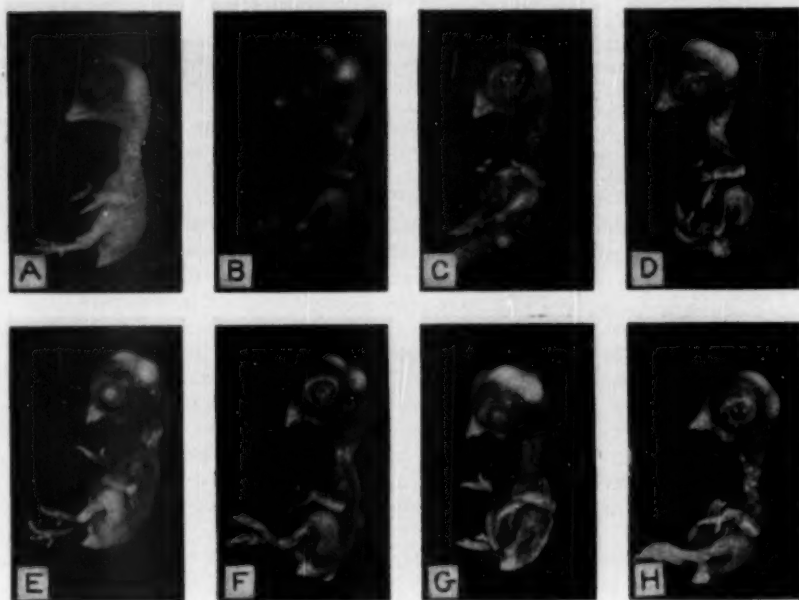


Fig. 3.—Chick embryos, nine days' incubation, showing same effects as in the embryos of 10 days' incubation shown in figure 2. A, normal; B, 2 hours; C, 6 hours; D, 10 hours; E, 14 hours; F, 18 hours; G, 22 hours; H, 48 hours.

incubation. With regard to the age of the embryo it must be emphasized that while every effort was made to ascertain the accurate age of the embryos, variations in incubation temperatures may alter embryonic development in some measure, and it is entirely possible that the estimated age levels may be in error by as much as 24 hours. Where large numbers of eggs are incubated for laboratory purposes it is a common experience that a certain number of dead embryos are encoun-

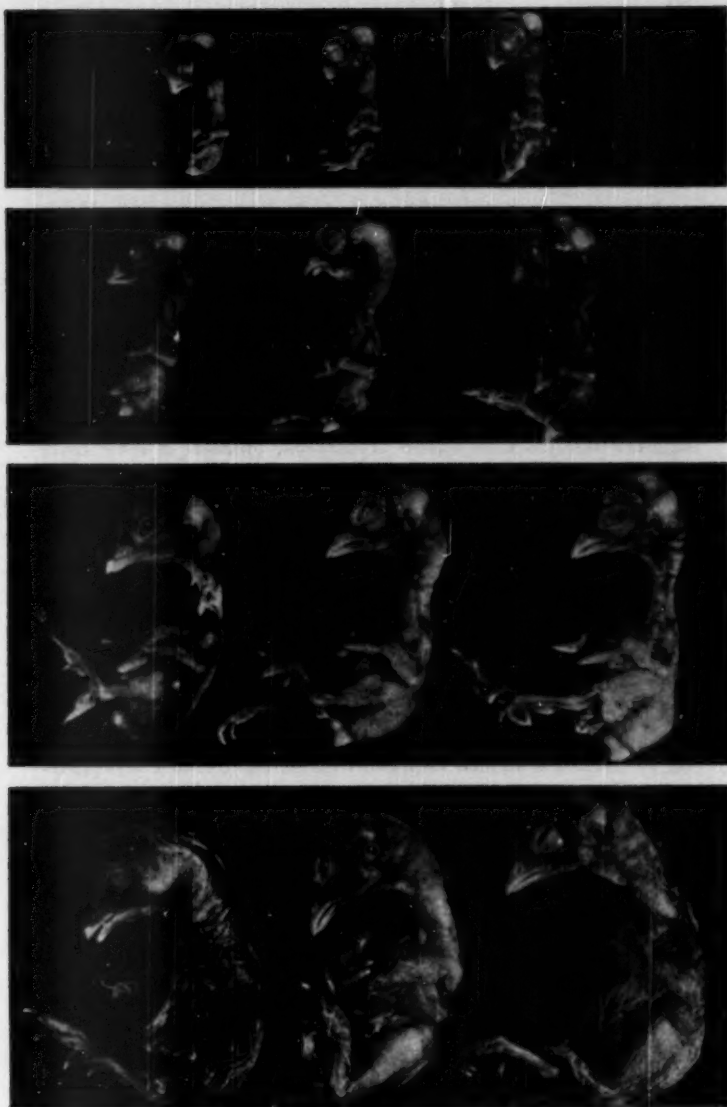


Fig. 4.—Effect of age of embryo on development of postmortem beak changes. Left to right in each row: 2 hours after death, 14 hours after death, 24 hours after death. First row: 8 day old embryos. Second row: 10 days old embryos. Third row: 12 day old embryos. Fourth row: 14 day old embryos.

tered in each incubation setting, the dead ones being eliminated as a routine procedure. These dead embryos occurring in the routine settings were noted by careful daily candling and were studied for postmortem changes. Dead embryos of 9, 10 and 11 days' incubation revealed the same abnormal beak changes as were seen in embryos of the same age which were killed by experimental methods.

Beak Changes Noted in Heads from Decapitated Embryos Transferred to Fluid Medium.—A number of experiments were carried out in an attempt to differentiate between actual growth of tissue after death and effects of autolytic processes. The question arose as to whether the beak abnormalities observed might possibly be the result of cellular proliferation, and whether in instances where the embryo circulation was interrupted experimentally the amniotic fluid might serve as a nutrient medium for continued local growth of some tissues. Local growth in dying or "dead" chick embryos has been reported,² and it has been suggested that the accumulation of metabolic products in some active tissue areas in the embryo might actually stimulate growth of adjacent tissue cells which are capable of utilizing nutrient substances from other neighboring tissues. However, the results of these studies seem to indicate that local cellular proliferation was not involved in the production of the beak changes observed.

In this connection one series of experiments was of particular interest. Aseptic precautions being used, the heads of 10 day old embryos were removed by clipping through the neck. The decapitated heads were washed in sterile Ringer's solution and dropped into a flask containing sterile Ringer's solution. The flask was placed in the incubator at 38 C. A control group of 10 embryos from the same egg setting was treated in an identical manner except that this flask was placed in the refrigerator at 15 C. In 24 hours the decapitated heads kept at incubator temperature showed the typical beak changes, whereas those kept in the refrigerator for a similar period showed no deviation from the normal appearance of live embryos of the same age.

The failure of the beak changes to occur within 24 hours under conditions of refrigeration might be considered as due to the retardation of cell division caused by lowered temperature or equally well explained on the basis that lowered temperature retards the processes of autolysis. Accordingly, a study was made of the effects of refrigeration on the development of beak changes.

Effect of Refrigeration on the Development of Beak Changes.—Two groups of seven embryos each were decapitated and the heads dropped into flasks of sterile Ringer's solution. Both groups were kept

2. Byerly, T. C.: Anat. Record **32**:249, 1926; **33**:319, 1926.

in the refrigerator for 24 hours at 15 C. At the end of this time none of the embryos in either group showed any beak changes. One group was allowed to remain in the refrigerator, while the other was placed in the incubator at 38 C. for another 24 hour period. The group which remained in the refrigerator showed no beak changes, whereas in the group which had been transferred to the incubator the typical beak changes developed.

To test further the effect of refrigeration on the retardation of the beak changes, 13 eggs 10 days old were coated with paraffin and placed in the refrigerator at 15 C. At intervals of two days an egg was opened and examined. By the fourth day after paraffin coating, slight changes were noted in the beak, with a slight suggestion of twisting of the upper beak. On the sixth day the embryo examined showed a definite short lower beak with the characteristic thickenings at the lateral posterior portion of the lower beak. Each egg opened after the sixth day showed the typical beak changes. On the tenth day the embryos showed indications of autolytic changes, such as dissolution of the epithelium over the brain surface. At 14 days the remaining six eggs were all opened. Each one showed the beak changes, and all showed marked autolytic changes of other tissues.

While cellular proliferation is considered possible even at temperatures as low as 9 C.,³ in the present studies all indications favored the conclusion that the underlying process in the development of the beak changes observed was autolytic in nature.

GROSS AND MICROSCOPIC EXAMINATION OF SPECIMENS

A careful measurement was made of the beaks of 10 embryos at 10 days of incubation, examined four hours after the paraffin coating of the shell. The embryos were dead, but no beak abnormalities were evident. The average length of the upper beak was 10.1 mm. and that of the lower beak 10 mm. At 24 hours after the paraffin-coating procedure, when all embryos showed the beak changes, measurement of 10 embryos of the same group showed the average length of the upper beak to be approximately the same (10.13 mm.), whereas that of the lower beak was only 7.13 mm., almost 3 mm. shorter than in those measured just after death. Careful examination of the under surface of the lower beak revealed that the decrease in length was compensated by an increase in width.

Such a phenomenon as this could occur if the supporting structures of the beak lost rigidity and became softened with resultant buckling. The bony structure of the beak in birds is derived from both cartilage

3. Stockard, C. R.: *Am. J. Anat.* 28:115, 1921.

and membrane bone. The main support of the lower beak is Meckel's cartilage, a V-shaped cartilage extending forward from either side of the lower jaw to an angular point at the extremity of the beak. Surrounding this cartilage are four membrane bones. In chicks of nine days' incubation, the mandible and the maxilla are just being laid down,⁴ and the ossification of the cartilage lags slightly behind that of the membrane bone. By the twelfth day these areas have expanded considerably.

These normal processes of ontogenesis were reviewed in an attempt to account for the changes observed after the death of the embryo. The conclusion seems reasonable that during this period of rapid beak growth between the ninth and twelfth days of incubation, the fragile segments of membrane bone present are not sufficient to hold the beak in its proper shape when the soft cartilages become distorted as a result of autolytic processes. This explanation of the beak change observed was substantiated when studies were made of whole embryos stained and cleared by differential staining for bone and cartilage according to the methods of Williams.⁵

The blue staining Meckel's cartilage, which is straight and rigid in fresh or recently dead embryos, stained very poorly in chicks dead 16 hours or more and appeared to be curved and buckled at the site of the "protrusions" or "thickenings" on the lower beak. Microscopic sections confirmed these observations. While facilities for serial sections were not available at this time, longitudinal sections were made at selected intervals through the beaks of five 10 day embryos which had been dead for 4, 8, 12, 16 and 24 hours, respectively. Transverse sections were made through the lower beak of one embryo which had been dead for 20 hours, and cross sections were made through the upper and lower beak of another embryo dead 20 hours. Controls were made on live embryos for each series of sections. In none of the sections was there any evidence of postmortem cellular proliferation. Evidence of autolysis was unquestionable in the dead embryos, and the degree of abnormality observed in the beak was in direct proportion to the progression of the autolytic processes. In live embryos and in those dead four hours or less, the cells appeared well stained and the cellular outlines were clearcut. By eight hours after death, autolysis had become apparent, and in these specimens bending and buckling of the cartilage were observed at the site of the lower beak protrusions, with associated tearing and disintegration of nearby connective tissue. As a result, wide spaces were present between the sagging cartilage and the adjacent reticulated bars of the developing membrane bones.

4. Lillie, F. R.: *The Development of the Chick*, ed. 2, revised, New York, Henry Holt & Company, Inc., 1940.

5. Williams, T. W., Jr.: *Stain Technol.* 16:23, 1941.

COMMENT

While there is probably little chance of misinterpretation of the more clearcut or obvious developmental anomalies, such as those reported by Franke and his co-workers⁶ as a result of injecting selenium salts into embryonated eggs, a word of caution is perhaps not out of order. Gross examination of embryos or fetuses should be correlated with careful microscopic studies in order to evaluate the significance of tissue and cellular changes. Differential staining is often of value. In examining any dead material for the presence of anomalies, one must differentiate growth disturbances from postmortem changes due to autolysis.

It is certainly possible that changes similar to the ones described in the chick embryo may occur also in other species. Byerly⁷ showed that actual disorganized cellular proliferation may take place locally in certain tissues in chick embryos of five days' incubation or less, even after the heart has stopped beating. The possibility that local disorganized growth may occur after circulation has failed is also suggested by the fact reported by Burrows⁷ that fibroblasts from chick embryos 4 to 5 days of age may grow for some time even in the absence of oxygen. It is well known that all cells do not die at the same time after the death of the organism as a whole. It seems reasonable that some cells may continue to grow after a fashion for some time, perhaps until supporting physiologic activities break down. Local tissue death or local occurrences interfering with cellular activities in crucial periods of development might be one factor involved in the production of anomalies observed in living embryos. In view of possible confusion arising from the effects of postmortem autolysis and possible postmortem cellular proliferation of an abnormal type, it seems important that in all experimental work concerned with developmental defects living material be harvested wherever possible.

SUMMARY

In normal chick embryos of 9, 10 and 11 days' incubation which have been killed and allowed to stand at incubator temperatures for eight to 16 hours after death, beak changes develop which may simulate those associated with developmental anomalies. These changes have been found in embryos dying of virus infection, in those dying after paraffin-coating of the shell, which interferes with respiration, in those dying as a result of hemorrhage from a clipped artery, decapitation and of unknown causes in the normal process of incubation. The beak defects are never found in live embryos or in embryos dead four hours or less.

6. Franke, K. W.; Moxon, A. L.; Poley, W. E., and Tully, W. C.: *Anat. Record* **65**:15, 1936.

7. Burrows, M. T.: *Proc. Soc. Exper. Biol. & Med.* **18**:133, 1920-1921.

but begin to make their appearance in embryos about eight hours after death, and are well developed by 16 hours after death. The changes occur in beaks of decapitated heads incubated for 24 hours in sterile Ringer's solution. Refrigeration at 15 C. retards the beak changes, but by the sixth day of refrigeration typical changes are observed. Embryos kept over night at 15 C. show no abnormal beaks, but the changes develop within 24 hours if the embryos are returned to the incubator at 38 C. Gross and microscopic examination of specimens failed to show any disorganized cellular growth. The deformity of the beaks is related to buckling and bending of the beak cartilages. Autolysis of the surrounding connective tissue was a consistent finding. While cellular proliferation occurring after death cannot be eliminated completely as a possible cause, the evidence seems to favor rapid autolysis of supporting structures as the most satisfactory explanation. Such postmortem autolysis offers a logical explanation also for the prominent beak abnormalities observed in normal embryos which die from unknown causes in the natural course of ontogenesis.

STUDIES ON AGING PROCESSES IN THE ENDOCRINE GLANDS OF THE GUINEA PIG

II. The Effects of Estrogen and Progesterone on the Thyroid, Parathyroid and Adrenal Glands of Male and Female Guinea Pigs of Various Ages

H. T. BLUMENTHAL, M.D.
ST. LOUIS

IN A PREVIOUS report¹ a parallelism was noted in the curves of diminution of the mitotic activity of the thyroid, parathyroid and adrenal glands with aging. However, at corresponding ages there was a greater rate of mitotic activity in female than in male guinea pigs; it was thought that this difference was at least partly genetically determined and independent of the activity of the female sex glands, since it was already present before the onset of sexual activity. On the other hand, experiments by Chouke, Friedman and Loeb^{2a} and by Chouke and Blumenthal^{2b} indicate a relation between proliferative activity of the thyroid and female sex hormones, since in the thyroid mitotic proliferation undergoes cycles which are correlated with the sexual cycle.

Recently Korenchevsky and co-workers³ have presented data and reviewed the experiments of other investigators which offer further evidence of a relation between ovarian secretions and proliferative activity of the thyroid and adrenal glands. These experiments deal with the effects of ovariectomy as well as with those of the administration of ovarian products. A summation of reports on the effects of castration indicate that in the adrenal gland there results an initial hyperplasia followed by a diminution of growth activity, while in the thyroid only a depression of proliferative activity occurs. An injected estrogenic substance may produce a transient enlargement of the thyroid, followed by definite evidence of atrophy. Korenchevsky also reports enlargement

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From the Departments of Pathology, St. Louis University School of Medicine, and the Jewish Hospital.

1. Blumenthal, H. T.: *Arch. Path.* **40**:264, 1945.

2. (a) Chouke, K. S.; Friedman, H., and Loeb, L.: *Anat. Rec.* **63**:131, 1935.
(b) Chouke, K. S., and Blumenthal, H. T.: *Endocrinology* **30**:511, 1942.

3. Korenchevsky, V., and Jones, V. E.: *J. Gerontol.* **2**:116, 1947; **3**:21, 1948.
Korenchevsky, V.; Paris, S. K., and Benjamin, B.: *Ibid.* **5**:120, 1950.

of the adrenal cortex following estrogen administration. On the other hand, progesterone (corpus luteum hormone) has been observed to produce atrophy of the adrenal⁴; there are reports of both stimulation⁵ and depression⁶ of the thyroid with this steroid.

Korenchevsky and co-workers have pointed out similarities between the effects of gonadectomy and changes occurring in senescence and have accordingly attempted to reverse these degenerative processes by injecting androgen, estrogen, progesterone and thyroid in various combinations. However, a diminution of the size of an endocrine organ or even histological evidence of atrophy may be no indication of the responsiveness of the gland to a hormonal stimulus. A young gland made atrophic by gonadectomy may be able to respond more readily to a given hormonal stimulus than an atrophic senescent gland. Thus, Korenchevsky has noted that in senescent rats the effects of endocrine products are in certain ways weaker than those obtained in adult gonadectomized rats. Therefore, in determining the endocrine status in senescence it is important not only to determine the changes in the circulating levels of stimulating substances with increasing age but also the changes in the ability of the target organ to respond to a given level of hormonal stimulus.

Accordingly, the present studies were carried out to determine the relation which age bears to the ability of the thyroid, parathyroid and adrenal glands to respond to estrogen and progesterone. We have also attempted to ascertain whether or not these glands respond in a parallel manner to estrogen and progesterone as they do to certain endocrine and chemical substances⁷ and under the influence of aging as reported previously.¹

MATERIALS AND METHODS

One hundred and seventy-eight guinea pigs were treated by injection of estrogen (estrone or alpha estradiol dipropionate in oil [progynon®], progesterone [proluton®]) or combinations of these.⁸ Many of the animals used in these experiments were the same as those used in a previous experiment reported by Blumenthal and Loeb.⁹

The mitotic activity of the thyroid, parathyroid and adrenal glands was determined according to methods previously described.¹⁰ The mitotic count of the cortex of the adrenal gland was recorded as the average number of mitoses per section, that of the thyroid as the average number of mitoses per gland (both lobes) and

4. Selye, H.: *J. A. M. A.* **115**:2246, 1940.

5. Alexiu, M.: *Arch. f. Gynäk* **100**:432, 1939.

6. Knaus, H.: *Arch. f. Gynäk* **110**:459, 1923.

7. (a) Blumenthal, H. T., and Loeb, L.: *Endocrinology* **30**:502, 1942. (b) Blumenthal, H. T.: *Ibid* **31**:226, 1942.

8. The estrogens and progesterone used in these experiments were supplied by the Schering Corporation, Bloomfield, N. J.

9. Blumenthal, H. T., and Loeb, L.: *Arch. Path.* **34**:49, 1942.

10. Blumenthal, H. T.: *Endocrinology* **27**:477, 1940. Blumenthal and Loeb.^{1a}

that of the parathyroid as the average number of mitoses per 10,000 cells. For the parathyroid the average number of epithelial cells per unit field was also recorded, since this figure is an index of the average cell size.

The guinea pigs were grouped according to weight at the termination of the experiment, each 100 Gm. of body weight serving as a division point. This division corresponds approximately to certain age groups, as pointed out in a previous report¹ and as shown in table 1.

The dose of estrogen or progesterone, the sex and the number of animals, as well as the specific procedures, are presented in the appropriate tables and in the section on results. Controls consisted of nontreated normal guinea pigs reported on previously or of gonadectomized animals of corresponding sex, age and weight; all castrated animals were 10 days past gonadectomy at the start of the experiments.

In the experiments in which estrogen was used some guinea pigs received small daily subcutaneous doses in distilled water (5 or 8 rat units), while others were given larger doses of a similar preparation (30 or 50 rat units). In still other experiments the guinea pigs received 200 rat units of alpha estradiol dipropionate in oil subcutaneously twice weekly; in its effect on the thyroid, parathyroid and adrenal glands the latter dose was indistinguishable from 50 rat units of the estrogen

TABLE 1.—*Weight-Age Groups*

Weight, Gm.	Age
100-150.....	4 days-1 mo.
200-300.....	1-4 mo.
300-500.....	4-8 mo.
600-800.....	8-12 mo.
900-1000.....	12-18 mo.
Over 1000.....	1.5-3 yr.

in water injected daily over a comparable period. The doses of progesterone administered varied between 0.2 and 1.0 international rabbit unit daily. The progesterone was injected subcutaneously in an oily suspension in all cases.

RESULTS

SERIES 1.—The effects of estrogen on the thyroid, parathyroid and adrenal glands of males and of nonovariectomized and ovariectomized females.

In these experiments the guinea pigs received subcutaneous injections of either 50 rat units of estrone in water daily or 200 rat units of alpha estradiol dipropionate in oil twice weekly. As stated previously, there was no significant difference between the effects of these two preparations in the doses administered, and the results were therefore combined.

The data in table 2 show that estrogen diminishes mitotic activity in the thyroid gland of the male guinea pig in all age groups, although only one middle-aged and one old guinea pig are included. The effect on mitotic activity in the parathyroid gland parallels that noted in the thyroid, although the epithelial cells of treated animals are, on the average, larger than those of controls. Adrenal glands were not available for study in this group. One animal in the youngest group, not included in table 2, received estrone for 56 days, at which time no mitoses were found in either the thyroid or the parathyroids. With all these guinea pigs there were such slight differences in individual counts that it was not necessary to include data on the range of variation in mitotic activity.

A similar experiment was carried out in nonovariectomized females, as shown in table 3. In immature females the injected estrogen diminishes the normal rate of mitotic activity in the thyroid and parathyroid glands; in the adrenal cortex mitotic activity is essentially unaffected in animals receiving injections for four to eight days but is moderately increased with longer periods of treatment. In young females at about the onset of sexual activity estrogen administered for four to

TABLE 2.—Male Guinea Pigs Receiving Either 50 Rat Units of Estrone in Water Daily or 200 Rat Units of Alpha Estradiol Dipropionate in Oil by Injection Twice Weekly

Guinea Pigs	Weight Range, Gm.	Days Treated	Mitotic Counts			
			Thyroid		Parathyroid	
			Exper.	Control	Exper.	Control
2	200-250	4-8	40.0	134.0	0.4-300	1.6-218.5
11	200-250	14-36	62.5	134.0	0.1-194	1.6-218.5
1	480	14	0	96.0	0-306	0.8-204.8
1	600	14	0	48.0	0-300	0.7-192.5
15						

TABLE 3.—Female Guinea Pigs (Nonovariectomized) Receiving the Same Doses of Estrone and Alpha Estradiol Dipropionate in Oil by Injection as the Males in Table 2

Guinea Pigs	Weight Range, Gm.	Days Treated	Mitotic Counts					
			Thyroid		Parathyroid		Adrenals	
			Exper.	Control	Exper.	Control	Exper.	Control
6	150-190	4-8	46.0	452.4	0.9-194	3.4-196	5.6	5.4
7	150-190	14-36	77.1	452.4	0.8-198	3.4-196	7.9	5.4
4	200-300	4-8	333.0	379.6	2.0-170	2.8-166	3.3	4.7
12	200-300	14-36	194.2	379.6	1.3-198	2.9-161	9.6	4.7
4	300-390	14-36	75.0	396.2	0.3-903	1.3-187	2.4	2.5
7	400-499	14-36	175.7	135.3	1.8-180	1.2-198	3.3	2.1
1	500-599	6	1,080.0	75.9	2.3-190	0.7-215
2	500-599	14-36	115.0	75.9	1.0-180	0.7-215
3	Over 600	4-8	230.0	97.5	1.3-187	0.6-214	1.0	0.2
3	Over 600	14-31	173.5	97.5	1.0-140	0.6-214
1	Over 600	5 months	0	57.5	0.3	0.2
30								

eight days has perhaps a slight inhibiting effect on all three glands; longer periods of treatment, however, produce a definite diminution of proliferative activity in the thyroid and parathyroid glands, but an increase is observed in the adrenal cortex. In progressively older groups the inhibiting effect on the thyroid and the parathyroid is gradually lost, and instead estrogen appears to stimulate mitotic activity. Judging from the results in the group weighing over 600 Gm., the stimulus is apparently most effective for periods up to about one week and then progressively diminishes. However, in one guinea pig which received estrogen for five months there was again an inhibition of mitotic activity in the thyroid gland, indicating the probability that resistance develops with prolonged injection of estrogen. The counts on the adrenal cortex are, on the average, only very slightly higher for the animals receiving estrogen than for controls, even with older animals.

In another experiment the influence of ovariectomy on the effects of estrone was tested. Four young mature (200 to 299 Gm.) guinea pigs were castrated; 10 days later they received 5 rat units of estrone for six days. The results were similar to those noted in corresponding animals with intact ovaries. Mitotic activity was plainly inhibited in the thyroid (30 mitoses per gland, on an average) and in the parathyroid (0.5 mitoses per 10,000 cells and an average field count of 202), but the adrenal showed a moderate increase in proliferative activity (7.7 mitoses per section). A similar experiment in four older castrated females (500 to 599 Gm.) showed complete absence of mitoses in all three glands; apparently the amount of estrone injected was too small to overcome the castration atrophy.

The inhibiting effect of estrogens in the younger age group was notable histologically in that the lining epithelium of the thyroid follicles was lower and the colloid harder in treated animals than in controls. In older experimental animals the epithelium was generally taller, but there was no essential change in the colloid or in the character of the connective tissue septums. As the data in table 3 illustrate, when proliferative activity is diminished in the parathyroid, the cells are generally

TABLE 4.—Male Guinea Pigs Receiving 0.65 to 1.0 International Unit of Progesterone by Injection Daily

Guinea Pigs	Weight Range, Gm.	Days Treated	Mitotic Counts					
			Thyroid		Parathyroid		Adrenals	
			Exper.	Control	Exper.	Control	Exper.	Control
3	150-199	7-14	546.6	104.2	2.5-212	2.6-301	8.1	3.1
3	200-299	7-14	878.7	134.0	3.8-198	1.6-219	9.5	2.8
3	300-399	42	226.7	134.0	1.3-170	1.6-219	15.0	2.5
9								

smaller, and when the average number of mitoses per 10,000 cells is increased, cell size is generally larger than in controls. In regard to the adrenal, no noteworthy histological differences have been noted between controls and animals receiving estrogen other than the changes in mitotic activity; the degree of change in proliferative activity was apparently not sufficiently great to produce a change in the thickness of the various zones, in cell size or in lipid content.

SERIES 2.—The effects of progesterone on the thyroid, parathyroid and adrenal glands of males and of castrated and noncastrated females.

The effects of subcutaneously injected progesterone on the mitotic activity of the thyroid, parathyroid and adrenal glands in immature and sexually mature young males is shown in table 4. In contrast to estrogen, this steroid greatly increases mitotic activity in the thyroids of these guinea pigs treated for seven to 14 days; animals receiving injections for a longer period show a similar but less striking response. On the other hand, the parathyroid glands of immature males are essentially unaffected by progesterone, but in young mature males the reaction parallels that noted in the thyroid gland; with prolonged injection there appears to be a moderate depression of proliferative activity. In both immature and mature males the adrenal cortex responds with increased mitotic activity and, unlike the thyroid and the parathyroid gland, shows a further increase of mitotic activity with prolonged administration of progesterone.

Two doses of progesterone were used in nonovariectomized females, as shown in groups A and B of table 5. The smaller dose, approximately one third that used in the males, appears to depress or not appreciably influence mitotic activity in all three glands in all age groups studied. In immature females the larger dose increases mitotic activity in all three glands. On the other hand, except for a slight increase of proliferative activity in one group of animals treated for 42 days, it appears to depress mitotic activity in the thyroid gland of the sexually mature animal; the thyroids of guinea pigs 4 to 8 months of age failed to respond even to prolonged injections of progesterone. The larger dose was without noteworthy

TABLE 5.—Female Guinea Pigs Receiving Progesterone

Guinea Pigs	Weight Range, Gm.	Days Treated	Mitotic Counts					
			Thyroid		Parathyroid		Adrenals	
			Exper.	Control	Exper.	Control	Exper.	Control
Group A. Nonovariectomized Female Guinea Pigs Receiving 0.30 or 0.85 International Units of Progesterone by Injection Daily								
1	150-199	11	100.0	452.4	2.1-190	3.4-196.2	6.8	5.4
4	300-399	6	110.0	379.6	2.7-200	2.8-163.2	7.7	4.7
4	300-399	12	100.0	295.2	1.1-186	1.3-187.1	2.8	2.8
7	400-499	6	173.3	135.3	1.9-190	1.3-196.1	2.8	2.1
15								
Group B. Nonovariectomized Female Guinea Pigs Receiving 0.65 or 1.0 International Units of Progesterone by Injection Daily								
7	150-199	6	648.4	452.4	4.9-131	3.4-196.2	6.4	5.4
4	300-399	7-14	298.0	379.6	2.2-180	2.8-163.2	7.6	4.7
4	300-399	42	460.0	379.6	2.7-186	2.8-163.2	21.7	4.7
2	300-399	6	100.0	295.2	1.4-186	1.3-187.1	3.4	2.8
3	300-399	56	100.0	295.2	0-645	1.3-187.1	16.3	2.8
19								
Group C. Ovariectomized Female Guinea Pigs Receiving 0.30 or 0.85 International Units of Progesterone by Injection Daily								
5	150-199	6	334.0	194.3	3.3-173	2.0-193	8.2	1.4
6	300-399	6	273.3	176.2	2.4-186	1.4-196	37.2	1.1
9	300-399	13	554.3	143.3	4.0-153	1.8-208	17.5	1.5
4	400-499	6	350.0	50.0	1.3-206	0.8-206	4.5	0.7
1	400-499	13	240.0	66.6	4.4	0.7
1	500-599	16	120.0	50.0	1.3	0.2
26								

effect on the parathyroid glands of mature female guinea pigs with intact ovaries except in the 4 to 8 month age group, in which prolonged injection completely suppressed mitotic activity. Increased proliferative activity of adrenal cortical cells was outstanding only in those animals receiving progesterone for long periods (42 and 56 days), and the response was somewhat higher in the younger of the two groups of mature animals.

The stimulating effect of progesterone in males and some sexually immature females in contrast to a depressing effect in young and middle-aged mature females might be explained in part by an antagonistic effect of the endogenously secreted estrogen of the latter animals. This was suggested by the occasional responses to prolonged injection of progesterone. Accordingly, a more extensive series of observations was made on ovariectomized animals, as shown in table 5, group C. Here the ability of progesterone to increase proliferative activity in the thyroid gland is seen in all age groups; in young mature guinea pigs 13 days of injection

gave higher mitotic counts than the shorter period of six days, but in older animals the reverse was true. In general there was a diminution in the degree of response with increasing age. Parathyroid glands were not available for study in all groups, but in those in which counts were made, the results paralleled mitotic counts obtained from the thyroid. The adrenal cortex also responded to administration of progesterone with increased mitotic activity, which again diminished with increasing age. The ovariectomized controls disclosed the depressing effects of castration on mitotic activity in all three glands. In general, the small dose of progesterone administered to group C was generally not sufficient to return mitotic activity to the precastration level except in the adrenal cortex and, in a few instances, in the thyroid and parathyroid glands.

Among the thyroids, an increase in the size of the epithelial cells lining the thyroid follicles and a softening of the colloid were noted in those glands showing

TABLE 6.—*The Effect of Estrone and Progesterone Injected into Nonovariectomized Female Guinea Pigs*

Guinea Pigs	Weight Range, Gm.	Days Treated	Mitotic Counts					
			Thyroid		Parathyroid		Adrenals	
			Exper.	Control	Exper.	Control	Exper.	Control
5 Rat Units of Estrone and 0.65 International Unit of Progesterone Daily								
2	150-199	6	0.7	5.4
2	200-299	6	300	279.6	0.3-216	2.5-103.2	11.5	4.7
5	300-399	6	231	225.2	7.7	2.9
14	400-499	6	196	135.7	1.9-196	1.3-196	4.3	2.1
3	500-599	6	133	73.9	1.5-160	0.7-215	2.9	2.0
1	Over 600	6	0	37.3	2.0	0.3
27								
200 Rat Units of Estrone Twice a Week and 1.3 International Unit of Progesterone Daily								
1	200-299	14	260	279	3.3-140	2.5-163
1	300-399	21	300	225	2.4-165	1.3-187
1	400-499	28	2640	135	4.0-152	1.3-196	23.3	2.1
2	400-499	5 months	45	135	2.0	2.1
1	Over 600	28	240	36	5.0	0.3
1	Over 600	5 months	80	56	0	0.2
7								

marked mitotic activity. In those instances in which the differences between experimental and control animals were not marked, histological differences were not noteworthy. Changes in the parathyroid cells as expressed in the figures denoting average field count consisted of an increase in cell size with increasing proliferative activity. The changes in the adrenal cortex were most striking. Most of the mitotic activity occurred either within the zona glomerulosa or at the junction of this area and the zona fasciculata. The changes in the lipid content of cortical cells with an increase in proliferative activity which has been described previously¹¹ as a response to other types of hormonal and nutritional stimuli were not striking in these experiments.

SERIES 3.—*The effects of combined injection of estrogen and progesterone on the thyroid, parathyroid and adrenal glands of noncastrated females.*

In the first group of animals small doses of estrogen and progesterone were given simultaneously to intact female guinea pigs for six days as shown in table 6.

There was a noteworthy increase of mitotic activity in the thyroid gland only in guinea pigs weighing between 400 and 599 Gm. In general, the degree of proliferative activity was not as great as that obtained with a smaller dose of progesterone in ovariectomized guinea pigs and resembles more the effect of estrogen on intact females. The results again demonstrate that older animals do not respond as readily as do younger ones, and in the oldest animal no mitoses were found. In the three groups from which parathyroids were available, the results essentially parallel those seen in the thyroid. The adrenal cortex shows some degree of stimulation in all groups except in immature animals; however, the degree of proliferative response again falls off with increasing age. On the whole, the response was not as great as with progesterone alone.

In the second experiment a larger dose of both steroids was used, and injections were carried out for a longer period. In the case of the one young, sexually mature guinea pig (200 to 299 Gm.) the thyroid count was slightly lower than that of the control, while the parathyroid showed an increase of mitotic activity. In the next group there was only a slight difference between the thyroid count of the treated animal and that of the control, but the parathyroid continued to show an appreciable proliferative response. A vigorous response was obtained in all three glands in a single animal in the next age group when injections were carried out for 28 days. It is interesting that this is the age group in which estrogen alone begins to produce a proliferative response. However, further prolongation of the injection period results in an inhibition of mitotic activity. Again, in the oldest age group there is still an appreciable but less impressive response in the thyroid and the adrenal after 28 days of injections, and this response is lost with prolongation of the injection period. These results indicate that the larger doses of estrogen and progesterone when injected for 28 days in guinea pigs weighing in excess of 400 Gm. (over 8 months of age) produce an increase in proliferative activity in the thyroid, the adrenal and probably also in the parathyroid (one guinea pig) greater than that produced by either substance alone.

COMMENT

The results of these experiments indicate that estrogen inhibits mitotic activity in the thyroid and parathyroid glands of young males and young females with intact ovaries, as well as in those of ovariectomized young and old females. On the other hand, it stimulates mitotic activity in nonovariectomized females if they are over 8 months of age; however, the degree of stimulation diminishes with further increase in age. The effect of estrogen on the adrenal cortex is not striking; there appears to be a moderate stimulation of mitotic activity in some instances, but no evidence of an inhibiting effect even in very young or very old animals. In general, mitotic activity also decreases with age in the adrenal cortex of estrogen-treated animals, in a manner parallel to that observed in controls.

The effects of progesterone are, in most instances, different from those of estrogen. The corpus luteum hormone acts as a mitotic stimulant on all three glands in young males (mature and immature) and in ovariectomized females of all age groups, with the exception of the parathyroid glands of sexually immature females. However, in mature

intact females progesterone appears to exert an inhibitory effect on mitotic division in all three glands, except that in the adrenal cortex after prolonged injection in an occasional group there may be no inhibition. In this respect the results are similar to those obtained with estrogen; but the results with castrated females show definitely that progesterone acts basically as a mitotic stimulant. In intact females the similarity to an estrogenic effect may be due to a state of hormonal imbalance; not only may the effects of progesterone be at least partially neutralized by endogenous estrogen, but the former may also inhibit the secretion of luteinizing hormone of the anterior lobe of the hypophysis, thus prolonging the life of large follicles in the ovaries, which could continue to secrete estrogen beyond the normal cyclic period. Therefore, the apparent inhibiting effect of exogenous progesterone might, in fact, be due to an increased production of endogenous estrogen. Such an explanation is consistent with the observations of Loeb¹² that the progesterone inhibits ovulation.

The combined injection of both these steroids in small doses produced an effect in the thyroid and parathyroid glands essentially similar to that observed with estrogen alone; apparently the combined endogenous and exogenous estrogens were sufficient in amount to offset any possible effect of progesterone. However, the adrenal cortex showed a degree of mitotic activity greater than that produced by estrogen alone, probably because both steroids have a stimulating effect on this gland. Furthermore, the adrenal cortex appears to be more sensitive to progesterone stimulation than the thyroid and parathyroid glands; this is substantiated by observations in several groups in which the adrenal responded to progesterone when the other two glands failed to show a proliferative reaction. Larger doses of both factors failed to produce an appreciable effect in animals under 8 months of age, but in animals over this age the effect of 28 days of injections was greater than that with either estrogen or progesterone alone.

In several of the experiments long periods of injections usually resulted in a lowering of mitotic activity. This lessening or inhibition of proliferative activity by hormones or hormone-like substances which stimulate mitotic activity when given for short periods has been previously noted by Loeb and co-workers.¹³

Loeb¹⁴ reported several antagonistic reactions between estrogen and progesterone. He has pointed out that the action of the corpus luteum

12. Loeb, L.: *Deutsche med. Wchnschr.* **37**:17, 1911; *J. Morphol.* **22**:37, 1911.

13. Gray, S. H., and Loeb, L.: *Am. J. Path.* **4**:257, 1928. Max, P.; Schmecke-bier, M. M., and Loeb, L.: *Endocrinology* **10**:329, 1935.

14. (a) Loeb, L.: *Am. J. Anat.* **32**:305, 1923; (b) *Proc. Soc. Exper. Biol. & Med.* **20**:443, 1923; (c) *Biol. Bull.* **27**:1, 1914. (d) Loeb, L., and Kountz, W. B.: *Proc. Soc. Exper. Biol. & Med.* **24**:728, 1928; (e) *Am. J. Physiol.* **84**:283, 1927.

not only inhibits ovulation but also the growth processes which estrogen calls forth in the vagina.^{14a,b} On the other hand, estrogen also inhibits the action of the corpus luteum by preventing the predecidual reaction of the uterine endometrium normally produced by follicular hormone.^{14c,d,e} Courrier¹⁵ also showed that in guinea pigs it is possible to prevent the formation of placentomas by injections of estrogen. The antagonism noted in the present experiments is similar to this second type. The stimulation of proliferative activity by progesterone in animals under 8 months of age can be inhibited by estrogen.

The selective stimulation of the zona glomerulosa by progesterone is particularly noteworthy. At the present time the controlling mechanisms of this zone are under investigation, owing primarily to the observations of Deane and Greep¹⁶ that there is a comparatively good maintenance of this zone in the rat after hypophysectomy. In fact, Deane, Shaw and Greep¹⁷ suggested that the zona glomerulosa actually becomes wider after ablation of the anterior lobe. On the other hand, Selye and Stone¹⁸ have been unable to confirm this observation. Sarason,¹⁹ as well as Deane and co-workers,¹⁷ have also reported that desoxycorticosterone causes a selective lipid depletion and involution of the zona glomerulosa, but Selye and Stone¹⁸ were also unable to confirm this observation. The latter investigators have noted that diets very rich in sodium chloride cause selective atrophy of the glomerulosa, while sodium-deficient diets produce hypertrophy of this zone; the latter observations are in agreement with the findings of Deane and co-workers¹⁷ as well as with those of Nichols.²⁰ We have been unable to find any previous reports of a stimulating effect of progesterone on the zona glomerulosa; on the contrary, Selye⁴ has reported that this agent produces atrophy of the adrenal cortex. Such differences point out the importance of utilizing several criteria in determining hyperplasia of endocrine organs. The fallacies of using weight or volume of endocrine glands as a means of determining reaction to various stimuli have been pointed out.²¹

Mechanisms controlling activity of the parathyroid glands are also under investigation. It is well known that the circulating levels of phosphorus and calcium play an important role in regulating the activity of these glands, but convincing proof of a parathyrotropic hormone of the hypophysis has not been established. In several previous investiga-

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16. Deane, H. W., and Greep, R. O.: *Am. J. Anat.* **70**:117, 1946.

17. Deane, H. W.; Shaw, J. H., and Greep, R. O.: *Endocrinology* **43**:133, 1948.

18. Selye, H., and Stone, H.: *On the Experimental Morphology of the Adrenal Cortex*, Springfield, Ill., Charles C Thomas, Publisher, 1950.

19. Sarason, E. L.: *Arch. Path.* **35**:373, 1943.

20. Nichols, J.: *Arch. Path.* **45**:717, 1948.

21. Blumenthal, H. T.: *Endocrinology* **27**:486, 1940.

tions we² have reported a parallelism in the reactions of the thyroid and parathyroid glands under various experimental conditions; in some of these experiments the adrenal cortex also reacted in a parallel manner. In the present experiments the three glands react similarly to progesterone, but there is a partial dissociation of this parallelism when estrogen is given. Under the latter condition the activity of the thyroid and parathyroid glands is depressed in young animals and stimulated in older ones while the adrenal cortex is either unaffected or mildly stimulated in all age groups. Up to the present time our investigations indicate that the thyroid and parathyroid glands react in a similar manner to hypophyseal preparations,^{2a} potassium iodide,^{2a,3} and ovarian products; they also show parallel changes in proliferative activity in aging¹ as well as in response to certain changes of nutritional status.²² The possible role of the thyroid in mediating response of the parathyroid glands is under further investigation.

Loeb²³ pointed out that the two principal types of structural changes which are characteristic of aging processes consist of (1) a gradual decrease of growth processes in various tissues, which may be accompanied by an increase in cell differentiation, and (2) hyalinization and sclerosis of stroma. Experiments have confirmed these observations and have given data quantitating the decrease of growth processes in the thyroid, parathyroid and adrenal glands in terms of changes in mitotic activity.¹ The present experiments represent an attempt to reverse these processes. In a general way they confirm the observations of Korenchevsky and co-workers² that estrogen, androgen and progesterone can at least partly increase growth processes in the glands of older animals, although generally the response decreases with increasing age. The observations reported here further point out that the sex hormones probably play a role in determining aging processes in these glands, although the role of the pituitary in regulating this effect remains to be evaluated more fully. There is some evidence that the secretion of pituitary trophic hormones diminishes with age, but some degree of hormonal stimulation can be produced even with the hypophyses of old animals.²⁴ In these experiments an interesting observation, not previously reported, is that the thyroid and parathyroid glands of female guinea pigs respond to estrogen only after the age of approximately 8 months. This observation requires further study and should be investigated also in other species. However, it may explain apparently contradictory reports concerning the effect of estrogen on the thyroid gland.

22. Blumenthal, H. T.: To be published.

23. Loeb, L., in Harvey Lectures, Baltimore, Williams & Wilkins Company, 1941, vol. 36, p. 228.

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SUMMARY

Estrogen in the doses used in these experiments depressed mitotic activity in the thyroid and parathyroid glands of male guinea pigs of all age groups. In nonovariectomized females it depressed mitotic activity in these glands up to about the age of 8 months, after which it stimulated mitotic activity. In two groups of gonadectomized females (one young, one old) estrogen failed to reverse the atrophying effects of castration or senescence on the thyroid and parathyroid glands. In the group of nonovariectomized females the injected estrogen either was without notable effect or produced a mild proliferative response in the adrenal cortex.

Progesterone in the doses used in these experiments increased mitotic activity in the thyroid and adrenal glands of immature and sexually mature young males and in the parathyroids of the latter. In nonovariectomized females, small doses depressed mitotic activity in the thyroid and parathyroid glands up to an age of approximately 8 months, while in the adrenal cortex there was very slight, if any, proliferative stimulus. In 8 to 12 month old nonovariectomized females the response was similar to that produced by estrogen. Larger doses of progesterone in nonovariectomized guinea pigs produced an increase in mitotic activity in the thyroid and parathyroid glands only in immature guinea pigs, but there was some degree of increase in proliferative activity of the adrenal cortex in all age groups. However, in ovariectomized guinea pigs progesterone produced an increase in mitotic activity in all three glands and in all age groups; this effect diminished in intensity with increasing age but was higher even than in nonovariectomized controls when corresponding age groups were compared.

In nonovariectomized females, small doses of estrone and progesterone produced an effect in all three glands similar to that observed with estrone alone. In larger doses the combination of these substances produced a proliferative effect in all three glands, and in some groups this exceeded that produced by either substance alone.

The histological observations made during the inhibition and during the stimulation of mitotic activity are described. Particularly noteworthy is the selective hyperplasia noted in the zona glomerulosa with injections of progesterone.

In those instances in which estrogen and progesterone induce hyperplasia, the effect is generally not as great as that produced by specific trophic hormones of the anterior lobe of the hypophysis. In older animals it is possible by proper dosage to produce a degree of proliferative activity comparable to that which normally occurs in younger age groups.

**STUDIES OF EARLY UTERINE CARCINOMAS DISCOVERED
BY CYTOLOGICAL EXAMINATION OF THE
VAGINAL CONTENTS**

**EDWARD L. BURNS, M.D.
WALTER H. HARTUNG Jr., M.D.
AND
ELIZABETH BRITTINGHAM, B.S.
TOLEDO, OHIO**

THE CYTOLOGICAL examination of vaginal contents has now become almost universally accepted as a reliable method for detecting cancers of the uterus, and many series of cases in which it has been done have been reported. A complete bibliography of the subject has been compiled in a recent monograph on the cytological diagnosis of cancer.¹ The uniformity of experience with regard to the type and the number of cases detected, the errors in diagnosis and the problems encountered in the operation of the program is impressive. Members of the medical profession in Toledo became interested in the possibilities of the method in 1946 and adopted a plan of operation in 1947.

The Toledo program is continuing to operate in the manner described in an earlier report.² The plan is similar in many respects to those functioning elsewhere, but at the same time it embodies certain unique features. It is sponsored by the local county medical society and is participated in by all members of the medical profession in Toledo. The smears are made in the offices of practicing physicians and from there are distributed to pathologists for examination. It is the aim to enroll all women in the program and particularly those without gynecologic symptoms or signs. Finally, and perhaps most important, a central registry is maintained by the County Medical Society from which notices are relayed through the physician's office to the patient asking her to return at intervals of six months to one year for repeated examinations. These notices are sent at regular intervals throughout the lifetime of the patient, and this feature gives to the program what we

From the Department of Pathology, Mercy Hospital.

1. Vincent Memorial Hospital, Boston: Cytologic Diagnosis of Cancer, Philadelphia, W. B. Saunders Company, 1950.

2. Hufford, C. E., and Burns, E. L.: Ohio State M. J. **44**:900, 1948.

feel is the primary requirement for early detection, that is, continuity of examination. This important point, we believe, is neglected in many plans.

Because of the system of repeated smears, opportunities are now developing for the study of smears from women in whom, during the course of our observations, carcinoma of the uterus appears to have developed. Because smears have been taken from many women who did not have gynecologic complaints, additional carcinomas have been accidentally discovered in an early state. It is the purpose of this paper to deal with these cases of early carcinoma, as well as to record briefly the results of the program as a whole.

RESULTS OF THE PROGRAM AS A WHOLE

These results, although taken from the material studied at only one hospital (Mercy), are representative of the entire program. From November 1946 to May 1950, 6,437 patients have been surveyed, and for these patients 9,205 smears have been made. Among the patients, 74 (1.14 per cent) have been found to have uterine carcinoma. The age range was from 26 to 81 years, with an average age of 48 years. Twenty of those with neoplasm (0.31 per cent of the 6,437 patients) had adenocarcinoma of the fundus; their ages ranged from 48 to 81 years, with an average age of 60 years. Fifty-four (0.83 per cent of the 6,437 patients) had squamous cell carcinoma of the cervix; 53 of these were between 26 and 73 years of age, with an average age of 46 years; the age of one patient was unavailable for this study. All neoplasms were demonstrated by histologic examination, and in many cases the entire cervix was cut into blocks and examined microscopically.

Thirty-six known errors were made in the examination of the smears. Calculated from the 372 biopsies of cervix or endometrium or both made in the survey, the percentage of error is 9.6. Exactly one half (18) of the errors were false negative diagnoses, of which 10 (2.7 per cent) were concerned with fundal and 8 (2.1 per cent) with cervical carcinoma. The remaining 18 errors represented false positive diagnoses. We have found it difficult to reduce the number of errors below this point. False negative diagnoses occurred more frequently in cases of advanced than in cases of early carcinoma, suggesting that the ulceration and infection associated with late cases may function to alter or destroy the neoplastic cells. It is in cases of this type that we have emphasized the necessity of biopsy for diagnosis regardless of smear findings. If this precaution is taken, no delay of diagnosis will result from false negative smear reports.

STUDIES OF CASES OF EARLY CANCER

Among the 74 cases of carcinoma of the uterus found in the survey there were 22 in which the presence of the neoplasm was not suspected

in the clinical evaluation of the patient. The determination of which were "unsuspected cases" was achieved by means of a questionnaire sent to the clinicians who first examined the patients. In many of these cases the patient had symptoms or findings which were undoubtedly due to the presence of the carcinoma, but the lesion was not sufficiently advanced to permit its being differentiated from non-neoplastic conditions which produce the same signs and symptoms. This observation reemphasizes the oft-repeated statement that early carcinoma cannot be distinguished by gross inspection from other non-neoplastic lesions of the uterus. In some of the "unsuspected cases," when biopsy was done, the carcinoma was found actually not to be early, and in those cases recur-

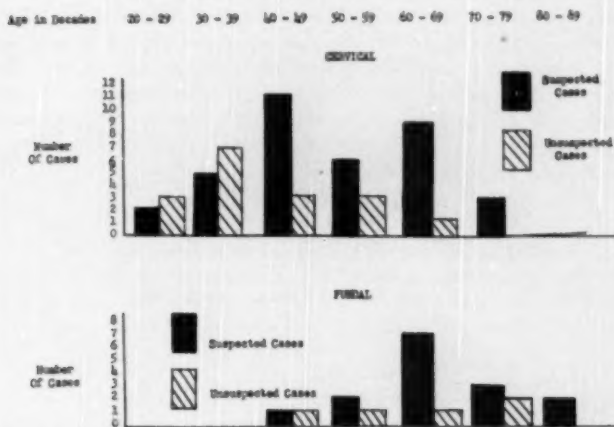


Fig. 1.—Age decade incidence of suspected and unsuspected carcinoma of the cervix and of carcinoma of the fundus of the uterus.

rence and death occurred. However, for purposes of analysis the entire group has been retained.

It was thought that if the "unsuspected cases" represented in general, a group of early uterine carcinomas, this fact might be manifested in the age of the patients. For this reason the age by decades and the average age found in the 22 "unsuspected cases" were compared with the same values in the 52 cases in which carcinoma was suspected clinically. The age range of the patients with unsuspected fundal carcinoma was from 49 to 71 years, with an average age of 61 years. The analysis by decades (fig. 1) shows the peak incidence (2 cases) between 70 and 79 years. The patients with suspected carcinoma showed an age range from 48 to 81 years and an average age of 66.3 years, and a peak incidence in

the decade of 60 to 69 years. This analysis does not strongly support the premise that the "unsuspected cases" of fundal carcinoma were discovered earlier than the "suspected cases," since the average age of the patients of the former group is approximately only seven years below that of the patients of the latter group, and the peak incidence falls one decade later.

In the "unsuspected cases" of cervical carcinoma the age range was from 26 to 61 years, with an average age of 39.8 years. The peak incidence (fig. 1) was between 30 and 39 years. In the "suspected cases," the age range was from 27 to 73 years, with an average age of 50.4 years. The peak incidence began between 40 and 49 years, with a second peak occurring in the 60 to 69 year group. The 10.6 year lower average age and the lower peak decade incidence in the "unsuspected cases" would support the conclusion that we are dealing with cases discovered at an earlier date than if the smear had not been used. Since the difference between the age groups is well marked, one might suspect that the transition from the "unsuspected" to the "suspected" stage was rather slow; in other words, that early carcinoma of the cervix may for a time grow rather slowly.

SURVIVAL RATES

Sufficient time has not yet elapsed for an accurate survey of survival rates. Rough analysis of the follow-up on the two groups as a whole, however, shows that in 75 per cent of the traceable "unsuspected cases" and in 45 per cent of the traceable "suspected cases" there is no evidence of recurrence.

CASES IN WHICH EARLY CARCINOMA WAS FOLLOWED BY SMEARS FOR LONGER PERIODS

Because of the practice of taking smears at regular intervals, a few opportunities have been presented for studying the changes in smear findings and the progress of cervical carcinoma over relatively long periods:

In case 17 the survey was conducted over a period of 2½ years. When the patient was first seen, Oct. 21, 1947, at the age of 33, she had irregular menstrual periods but otherwise no complaints. Pelvic examination showed a slight cystocele, a stellate laceration of the cervix and a raised area 0.5 cm. in diameter on the cervix. Study of exfoliated cells, as well as biopsy of cervix and endometrium, in 1947 did not show neoplastic or preneoplastic changes. Routine smears made at six month intervals in 1948 likewise failed to show cells suggestive of neoplasm. In 1949, however, two smears made at six month intervals showed atypical cells, which could not be positively identified as neoplastic. The first unequivocal cells were found in a smear made in February 1950, and a smear taken one month later also showed neoplastic cells. A biopsy specimen taken in April 1950 showed an early squamous cell carcinoma of the cervix (figs. 2 and 3).

In case 6 a carcinoma was followed for a period of one year. When first seen, the patient, a 39 year old woman, complained of bleeding and discharge. Pelvic examination showed a cervical polyp and cervical ulcers which bled easily. The first smear, made in October 1946, showed neoplastic cells (fig. 4A). The patient was advised to enter the hospital for biopsy, but refused. A punch biopsy, done in the physician's office at that time, gave negative results. The patient was next seen 11½ months later. She had no complaints, but pelvic examination showed a "suspicious" area in the cervix. A vaginal smear again showed neoplastic cells (fig. 4B). Hysterectomy was performed, and multiple sections of the cervix showed a single focus of early invasive squamous cell carcinoma (fig. 5).

In case 16 the neoplastic change was followed for a period of 9½ months. When first seen, the patient, a 28 year old woman, had no complaints and visited her physician for a routine postpartum check-up. The cervix was lacerated but other-

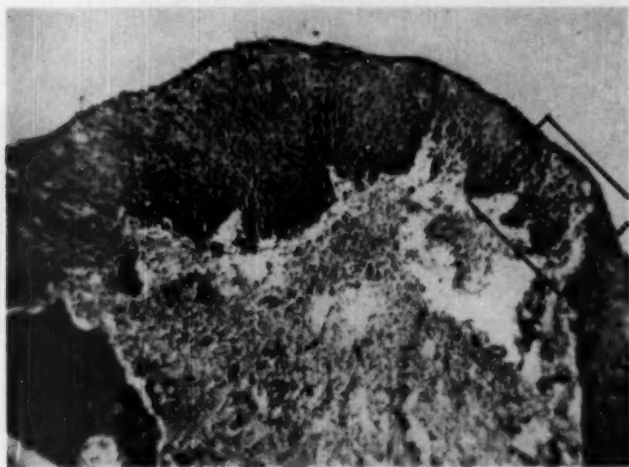


Fig. 2 (case 17).—Intraepithelial squamous cell carcinoma first discovered by vaginal smear. Hematoxylin and eosin stain; $\times 110$.

wise showed no changes. Neoplastic cells, however, were found in the vaginal smear made at that time. She refused reexamination until 9½ months later. Again neoplastic cells were found in the vaginal smear. Her cervix was conized, and histologic studies showed an intraepithelial carcinoma.

These three cases, while few, are interesting from the standpoint of the possible genesis of cervical carcinoma. In case 17 the neoplastic cells were present in smears for approximately two months before an early carcinoma was histologically demonstrated, and atypical cells, not positively identified as neoplastic, were present for approximately 12 months before neoplastic cells were found. In cases 6 and 16 the neoplastic cells were found in the vaginal smears 12 and 9½ months,

respectively, before the carcinoma was histologically demonstrated. Since the neoplastic cells were present when smears were first made in these cases, it must be assumed that in the cervical epithelium the neoplastic changes had extended over a longer period than that indicated by the smear. In each of the cases the histologic studies showed either intraepithelial carcinoma or early invasive carcinoma. These findings

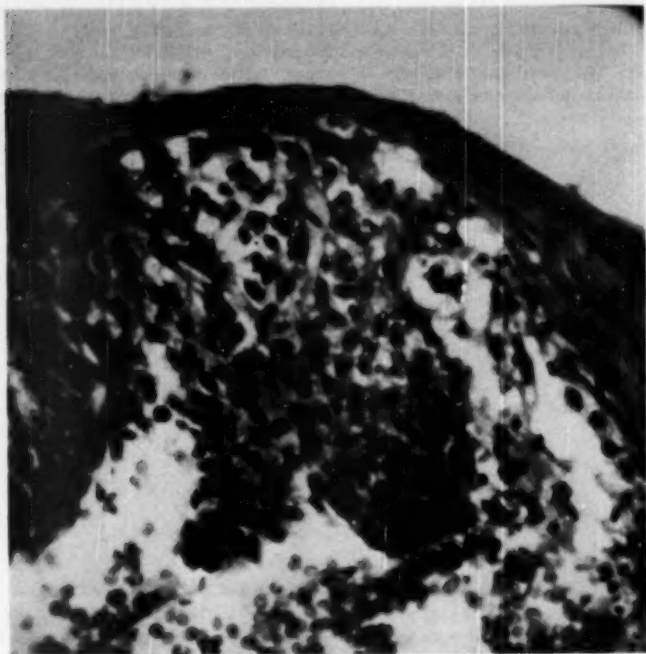


Fig. 3 (case 17).—Higher magnification of the field indicated in figure 2. Note the strand of normal epithelium extending from the right. The cell layers become disorganized in the central area of the abnormal proliferation. Hematoxylin and eosin stain; $\times 485$.

suggest, as other studies³ have suggested, that in the development of some invasive cervical cancers there is a latent period during which carcinomatous changes develop in, and are limited to, the cervical epithelium. If further studies confirm these observations, this fact combined with the greater accuracy of smear diagnosis in the early

3. Schiller, W.: *Am. J. Obst. & Gynec.* **34**:365, 1937. Knight, N. D.: *Ibid.* **46**:333, 1943.

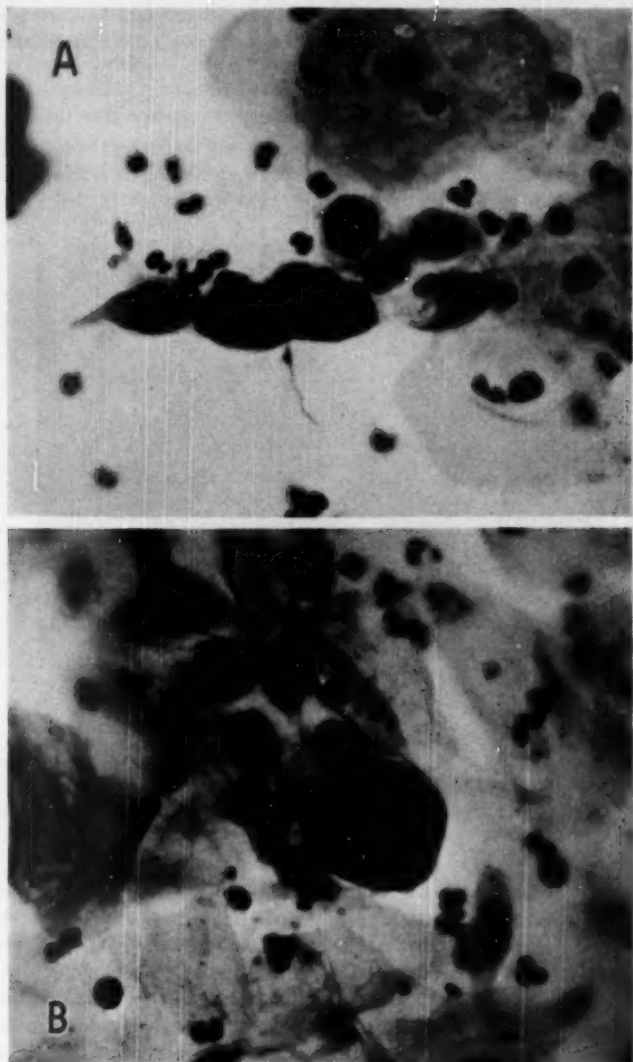


Fig. 4 (case 6).—*A*, neoplastic cells found in vaginal smear one year before the histologic section (fig. 5) demonstrated an early squamous cell carcinoma; *B*, neoplastic cells in vaginal smear made 11½ months later than that from which *A* was prepared. Papanicolaou EA 31 stain; $\times 725$.

cases will make routine cytological examinations of vaginal contents an even more effective measure for detecting early uterine carcinoma than had heretofore been expected.

HISTOLOGIC CHANGES IN CERVIXES SHOWING EARLY CARCINOMA

In nine cases the finding of neoplastic cells in vaginal smears has led to the detection of cervical carcinomas which were considered to be early enough to warrant treatment by total extirpation of the uterus.

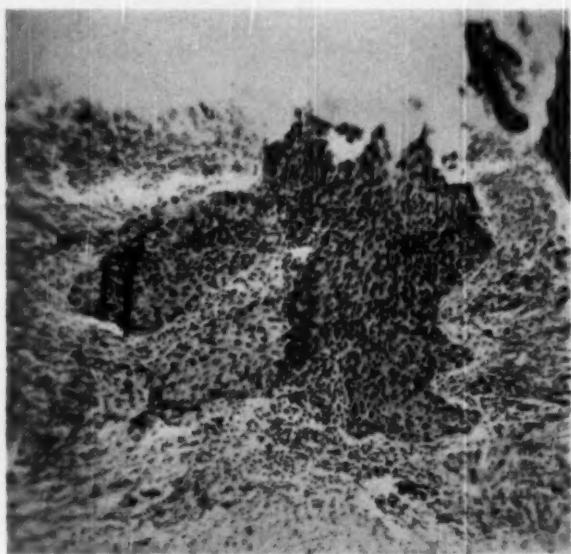


Fig. 5 (case 6).—The single area of early invasive squamous cell carcinoma found after complete sectioning of the cervix. Hematoxylin and eosin stain; $\times 110$.

These cases have offered special opportunities for studying the distribution of atypical epithelial changes in early cervical carcinoma. In four of these nine cases the early carcinomatous changes were found in single, limited, invasive or noninvasive foci in the cervical epithelium. In five cases, however, atypical changes were found in multiple locations, sometimes in widely separated areas. In one case slight changes were noted even at the margin of the vaginal cuff where the cervix was surgically removed from the vagina (fig. 6). These changes consisted in disorganization of the normal layers of the stratified squamous epithelium.

in development of large cells with hyperchromatic nuclei showing mitotic figures and variation in size, and in thickening of the epithelium, with or without processes extending into the underlying connective tissue, which invariably contained lymphocytes (fig. 3). These findings are in accord with experimental observations in animals in which it has been shown that generalized growth changes may occur before localized invasive processes develop.⁴ Whether invasion, when it occurs, proceeds from many or few such areas of altered epithelium is still to be studied. Such findings suggest the possibility that carcinoma may develop in the vaginal cuff after total extirpation of the uterus and emphasize the need for follow-up study of patients so treated. It is our opinion that such follow-up studies should include the regularly repeated examination of vaginal smears.

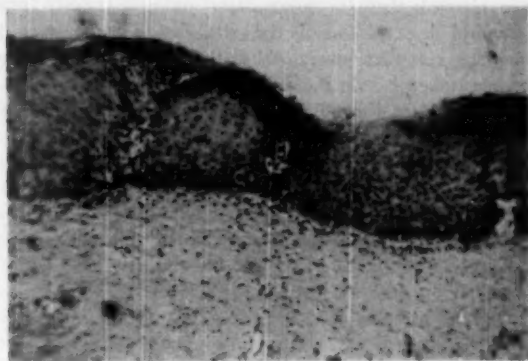


Fig. 6 (case 13).—Atypical changes in stratified squamous epithelium found extending to excised margin of vaginal cuff in a case of early carcinoma of the cervix. Hematoxylin and eosin stain; $\times 110$.

SUMMARY

The Toledo program for early detection of uterine cancer embodies an area-wide plan of making a pelvic examination and a cytological examination of the vaginal contents of every woman at regular intervals of six months to one year. This plan gives continuity to the detection program, a feature which we feel is of prime importance and usually neglected.

Of 6,437 patients surveyed at one hospital, 74 (1.14 per cent) were proved to have uterine carcinoma.

4. Suntzeff, V.; Burns, E. L.; Moskop, M., and Loeb, L.: *Am. J. Cancer* 32:256, 1938.

Thirty-six known errors have been made in the examination of the 9,205 smears made from the 6,437 patients.

Twenty-two of the 74 shown to have uterine carcinoma were not suspected of having it from clinical symptoms or the results of pelvic examination, and the first evidence of neoplasm was given by the vaginal smear.

Studies of age incidence in the 22 "unsuspected cases" as compared with the 52 "suspected cases" show the average age of patients and the decade incidence of carcinoma of the cervix to be lower in the "unsuspected group." The possibility of a latent period, during which in some cases carcinoma of the cervix grows slowly and perhaps remains localized to the epithelium, is discussed.

Histologic studies on nine uteri removed for early carcinoma of the cervix showed that in some cases multiple widely separated foci of atypical hyperplasia of the stratified squamous epithelium occur. This suggests that generalized disturbances of growth processes may sometimes take place in the cervix before neoplasia develops. The significance of this fact, if confirmed, with regard to early diagnosis and recurrence after treatment is discussed.

STUDIES IN AMINO ACID UTILIZATION

Tissue Protein Synthesis as Influenced by the Dietary Intake of Essential Amino Acids

PAUL R. CANNON, M.D.
LAURENCE E. FRAZIER, M.S.
AND
RANDOLPH H. HUGHES
CHICAGO

THERE is accumulating evidence from experiments on intact animals that in the synthesis of tissue protein the over-all processes of protein metabolism are so active as to require the simultaneous availability of all the essential amino acids.¹ This seems to be true both for growth and for maintenance of nitrogen balance and weight. Evidently the processes of protein synthesis are both rapid and "perfectionistic," judging from experiments in which delayed administration of one or more essential amino acids leads to impairment of synthesis. In short, in the presence of an acute essential amino acid deficiency these rapidly functioning mechanisms presumably allow too little time for tissue enzymes to release adequate quantities of a particular essential amino acid, or the enzymes do not act quickly enough, for it to be fitted into the synthesizing patterns. In consequence catabolism develops rapidly, as indicated by the ensuing negative nitrogen balance. Evidence to date suggests that this is the case in both rats and men.²

In earlier experiments we observed that if any one of nine indispensable amino acids was omitted from a ration otherwise adequate in essential nutrients the lack of it caused protein-depleted rats quickly to

From the Department of Pathology, University of Chicago.

This work was done in cooperation with the Navy Department Office of Naval Research. We also acknowledge the cooperation of the Douglas Smith Foundation for Medical Research of the University of Chicago.

1. Berg, C. P., and Rose, W. C.: *J. Biol. Chem.* **82**:479, 1929. Elman, R.: *Proc. Soc. Exper. Biol. & Med.* **40**:484, 1939. Geiger, E.: *J. Nutrition* **36**:813, 1948, **34**:97, 1947. Cannon, P. R.; Steffee, C. H.; Frazier, L. E.; Rowley, D. A., and Stepto, R. C.: *Federation Proc.* **6**:390, 1947. Wissler, R. W.; Steffee, C. H.; Frazier, L. E.; Woolridge, R. L., and Benditt, E. P.: *J. Nutrition*, **36**:245, 1948. Henderson, R., and Harris, R. S.: *Federation Proc.* **8**:385, 1949. Geiger, E.: *Science* **111**:594, 1950. Cannon, P. R.: *Federation Proc.* **7**:391, 1948.

2. Rose, W. C.: *Ibid.* **8**:546, 1949. Leverton, R. M., and Gram, M. R.: *J. Nutrition* **39**:57, 1949.

lose appetite and weight.³ When the missing amino acid was restored to the ration, the animals regained both weight and appetite. From these experiments it was obvious that acute amino acid deficiency disease develops rapidly in the dietary absence of a single indispensable amino acid. However, the concomitant development of caloric deficiency, while accentuating the adverse consequences of the amino acid lack, also complicated interpretation of the experiments. A later study by Wissler and associates⁴ clarified the situation to some extent by demonstrating that negative nitrogen balance and loss of weight accompanied essential amino acid deficiency despite an adequate caloric intake accomplished by forced feeding.

It seemed desirable, however, to repeat our earlier experiments by means of a method whereby all dietary nitrogen could be given in fluid form, with a separate basal ration supplying calories, vitamins and salts. In that way an acute amino acid deficiency could be produced without alteration of the basal ration, thus ruling out factors of taste or odor which might otherwise influence acceptance of the latter dietary component.

METHODS

Protein-depleted adult male albino rats (Sprague-Dawley), originally weighing from 200 to 210 Gm., which had lost from 25 to 30 per cent of their predepletion weight through ingestion of a low protein diet⁵ were placed in individual cages and fed a protein-free basal ration supplemented with an amino acid solution supplied in drinking fountains. The basal ration contained the following ingredients: dextrin, 67.8; corn oil, 4.4; salt mixture (Osborne and Mendel, Hawk and Oser modification), 4.4; ruffex,⁶ 5.6; vitamin mixture, 1.1; oleum percomorphum, 0.3 drop; water, 16.7. The vitamin mixture had the following percentage composition: choline, 20; nicotinamide, 0.426; calcium pantothenate, 0.263; pyridoxine hydrochloride, 0.096; riboflavin, 0.162; thiamine hydrochloride, 0.081; sucrose, 79.315. Because of its insolubility, tyrosine was also added to the basal ration, from 84 to 98.5 mg. per rat day, depending on whether solution B or solution A was being used. This ration, in the amounts of 13.5 Gm. per day, had a caloric potentiality of approximately 42 nonprotein calories. For three days before an experiment was started, each rat was fed our "protein-free" 4E ration,⁵ with water supplied in the drinking fountains, in order to accustom him to the experimental regimen.

The amino acid solution first used was designated solution A. It was essentially a 5 per cent solution, and in amounts of 35 ml. per day it furnished approximately 208 mg. of nitrogen. This plus the tyrosine in the basal ration gave a "protein" content of approximately 1.35 Gm. per day. This solution was used to evaluate the effects of administering varying amounts of tryptophan, histidine, phenylalanine

3. Frazier, L. E.; Wissler, R. W.; Steffee, C. H.; Woolridge, R. L., and Cannon, P. R.: *J. Nutrition* **33**:65, 1947.

4. Wissler, R. W.; Frazier, L. E., and Slayton, R. E.: *Proc. Soc. Exper. Biol. & Med.* **72**:589, 1949.

5. Wissler, R. W.; Woolridge, R. L.; Steffee, C. H., and Cannon, P. R.: *J. Immunol.* **52**:267, 1946.

and methionine. For the other six amino acids studied a second amino acid solution, designated solution B, was used. This solution contained approximately a 10 per cent increase in the amounts of each of nine indispensable amino acids but with the over-all nitrogen content unchanged. It was designed to improve to some degree the nutritive potentiality of the amino acid solution. No water was given during the experimental periods, in order to encourage complete consumption of the amino acid solution. The composition of these two solutions is given in table 1.

In a recent communication⁶ we presented figures indicating the minimal amounts, expressed as utilizable forms, of each of nine essential amino acids required in our standard amino acid mixture to insure a weight recovery of 40 Gm. in 10 days in a protein-depleted rat weighing initially 144 Gm., having a mean repletion weight of 165 Gm. and consuming 95 per cent of the ration. It was pointed out, however, that these findings had no absolute value except under the experimental conditions described. It should also be emphasized that these values were obtained under

TABLE 1.—Composition of the Amino Acid Solutions Used

	Percentage Composition		Mg. in 35 Ml.		Minimal Amounts of Essential Amino Acids, in 35 Ml., Mg.
	Solution A	Solution B	Solution A	Solution B	
DL Alanine.....	5.01	4.23	86.1	73.5	86.5
L Arginine HCl.....	4.44	3.75	76.3	65.3	76.7
DL Aspartic acid.....	5.64	4.77	97.0	82.3	97.4
L Glutamic acid.....	21.31	17.96	365.0	311.8	386.5
.... Glycine.....	0.45	0.38	7.7	6.6	7.8
L Histidine HCl.....	3.03	3.38	51.8	57.0	59.0
DL Isoleucine.....	11.61	12.67	200.0	220.0	132.0
L Leucine.....	10.82	11.79	186.3	204.8	73.5
L Lysine HCl.....	8.47	9.20	145.6	160.2	79.5
DL Methionine.....	3.53	3.84	60.6	66.7	39.0
DL Phenylalanine.....	4.65	5.06	80.3	88.3	45.0
DL Threonine.....	6.98	7.6	120.0	132.0	86.0
DL Tryptophan.....	1.63	1.76	27.7	30.5	14.5
L Tyrosine.....	Included in basal diet				
DL Valine.....	12.50	13.66	215.0	237.2	108.0

conditions of simultaneous ingestion of amino acids and other dietary components and might not be expected to apply under the conditions of the present experiments in which amino acids in solution were imbibed with no sharp relationship to ingestion of the basal ration. Under such circumstances it would not be surprising if some of these "minima" should be subminimal, and, as will be seen, such was the case with some. Nevertheless the present experiments afford a further check on the validity of the "minima" which we previously established under a different set of experimental conditions.

EXPERIMENTAL FINDINGS

Experiment 1: Tryptophan.—The effects incident to variations in the daily intake of tryptophan were determined in 20 protein-depleted rats over a period of 10 days (table 2). These effects were measured

6. Steffee, C. H.; Wissler, R. W.; Humphreys, E. M.; Benditt, E. P.; Woolridge, R. L., and Cannon, P. R.: *J. Nutrition*, **40**:483, 1950.

in terms of consumption of the amino acid solution and of the basal ration and in terms of weight recovery. Inasmuch as we have found as a result of many carcass analyses that during repletion there is a close correlation between gain in body weight and deposition of body nitrogen, the extent of weight recovery is an excellent indicator of tissue refabrication, largely of muscle mass. It should be pointed out, also, that the repletion ration is a low fat ration and in 10 days the formation of adipose tissue is not great.

When solution A containing 27.7 mg. of tryptophan per 35 ml. was offered, there was practically complete acceptance of both the solution and the basal ration, with an average weight recovery of 49 Gm. per rat (group 1). When the tryptophan content was reduced to 14.5 mg. per 35 ml. the average weight recovery was 40 Gm. per rat (group 2). With a tryptophan content of 7.25 mg. per 35 ml. there was a decline in consumption of both food and drink and in the 10 days the five animals

TABLE 2.—*Influence of Variations in Intake of Tryptophan on Consumption of Food, Drink and Weight Over a Period of Ten Days*

Group	Rats	Amino Acid Solution, 35 Ml. per Day	Average Percentage Consumption of Solution	Average Percentage Consumption of Basal Ration	Average Weight Gain (or Loss), Gm.
1	4	Solution A with 27.7 mg. of tryptophan	98	97	49 ± 2.8
2	2	Solution A with 14.5 mg. of tryptophan	100	96	40 ± 0.47
3	5	Solution A with 7.25 mg. of tryptophan	13	46	-30 ± 6.9
4	5	Solution A without tryptophan.....	11	44	-30 ± 2.5
5	4	Solution A with essential amino acids in minimal amounts.....	90	79	31 ± 0.67

lost an average of 20 Gm. per rat (group 3). This average loss of weight was essentially the same as that of the rats which received no tryptophan at all (group 4). Finally, when a group of rats (group 5) received solution A containing the nine essential amino acids at minimal levels the average weight recovery was only 31 Gm. per rat. It is obvious, therefore, that the absence of only a few milligrams of tryptophan below minimal requirements led to a marked impairment of tissue refabrication as expressed in terms of weight recovery.

Further data in relation to tryptophan intake are presented in charts 1 and 2. Thus a protein-depleted rat receiving solution A and the basal ration for four days (chart 1) consumed both dietary components and made a weight gain of approximately 25 Gm. After tryptophan was removed from solution A on the fifth day the rat drank only about two thirds of the solution, although eating practically all the basal ration. During the next three days, however, he drank only a few milliliters of the solution each day, although continuing to eat more than one-half the basal ration. Nevertheless his weight declined. With tryptophan

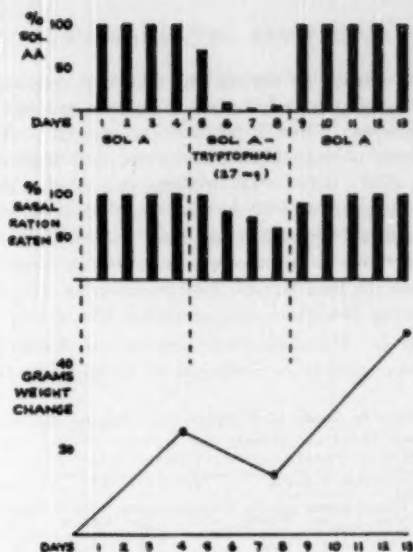


Chart 1.—Influence of tryptophan deficiency on a protein-depleted rat's acceptance of food and drink. (Rat 312-6-1-12D.)

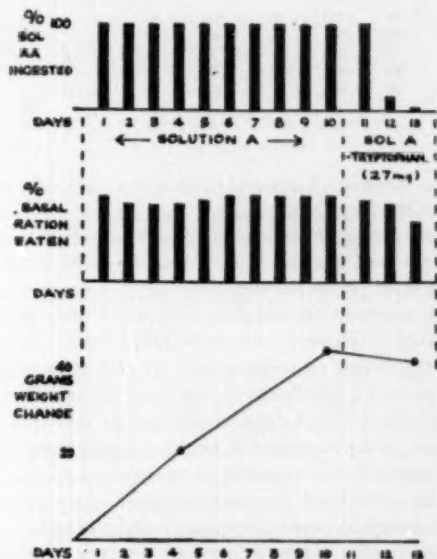


Chart 2.—Influence of tryptophan deficiency on a protein-depleted rat's acceptance of food and fluid. (Rat 313-7-1-12D.) The 100 per cent level and the days of observation are the same for the basal ration as for the solution.

restored to the solution for the ensuing five days, consumption of both the ration and solution A again became practically complete and recovery of weight was rapid. Chart 2 indicates that the rat's refusal to drink solution A devoid of tryptophan was not simply a matter of a change in its taste or odor. Thus a rat drinking this solution and eating the basal ration over a period of 10 days continued to do so on the eleventh day, even though all tryptophan had been removed from the solution. During the next two days, however, he refused to drink most of the solution, and on the final day his food consumption dropped off about one third. During these three days, moreover, loss of weight ensued.

Experiment 2: Histidine, Phenylalanine and Methionine.—In the second experiment, and in the remainder of the experiments, the feeding

TABLE 3.—*Influence of Variation in Intake of Histidine, Phenylalanine and Methionine on Consumption of Food, Drink and Weight over a Period of Ten Days*

Amino Acid	Daily Amount in 35 Ml., Mg.	Rate	Period 1 (3 Days)			Period 2 (4 Days)			Period 3 (3 Days)			Weight Gain (Total), Gm.
			Liquid Consumed, %	Ration Consumed, %	Weight Gain, Gm.	Liquid Consumed, %	Ration Consumed, %	Weight Gain or Loss, Gm.	Liquid Consumed, %	Ration Consumed, %	Weight Gain, Gm.	
Histidine.....	51.8	4	94	99	19	100	100	17	100	99	11	47 ± 1.4
Histidine.....	29	5	97	99	18	84	96	15	100	99	8	41 ± 1.4
Histidine.....	14.5	5	91	99	20	25	89	-9	81	86	26	37 ± 2.7
Histidine.....	0.0	4	96	97	19	16	78	-20	80	87	30	25 ± 1.8
Phenylalanine.....	80.2	6	93	97	20	100	100	21	100	100	9	50 ± 1.0
Phenylalanine.....	45	6	100	100	19	100	96	16	100	100	10	45 ± 1.73
Phenylalanine.....	22.5	6	99	98	21	72	70	-5	95	77	16	38 ± 2.04
Phenylalanine.....	0.0	6	100	99	22	18	70	-21	79	92	24	35 ± 1.42
Methionine.....	60.6	4	96	96	18	100	99	18	100	99	11	47 ± 2.35
Methionine.....	30.3	5	98	99	23	100	100	13	100	100	11	47 ± 2.01
Methionine.....	0.0	5	98	99	20	26	80	-14	100	100	25	31 ± 1.0

procedure was altered as follows: For three days all rats received solution A and the basal ration (period 1). During the next four days (period 2) one group of animals continued to receive solution A, a second group received solution A containing the minimal daily allotment of histidine, a third group received solution A containing one-half the daily minimal allotment of histidine, and the fourth group received solution A devoid of histidine. All continued to receive the basal ration. At the end of this period all animals again received solution A for a final three days (period 3), together with the basal ration. The results are summarized in table 3. Here it can be seen that the rats drinking solution A for the entire 10 days accepted it practically completely; they ate the basal ration completely and achieved an average weight gain of 47 Gm. per rat. On the other hand, the group supplied with the minimal allotment of histidine during period 2 consumed only about 85 per cent of the solution and averaged a weight gain for the 10 days of 41 Gm. At the

same time the rats which received only one-half the daily minimal allotment of histidine consumed only 35 per cent of the amino acid solution, and their acceptance of the basal ration was less than 90 per cent. As a consequence their weight gain for 10 days was 37 Gm. Finally, the animals receiving no histidine during period 2 drank only 16 per cent of the solution, they ate less than 80 per cent of the basal ration and lost an average of 20 Gm. per rat. Despite an improvement

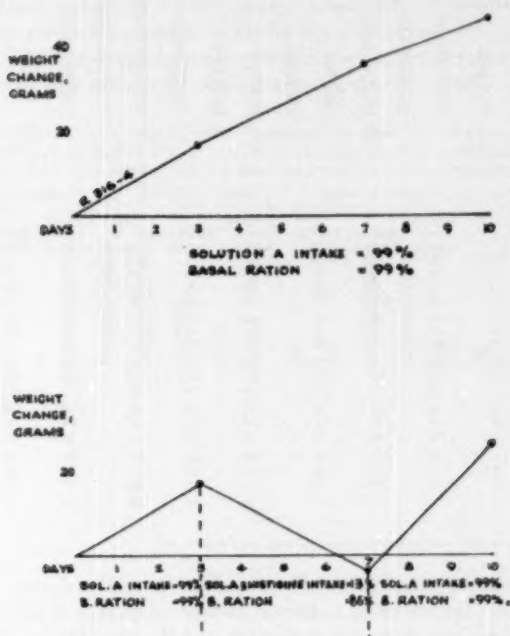


Chart 3.—Influence of histidine deficiency on a protein-depleted rat's protein repletion. (Rat 316-4.) The upper graph shows a control curve; the lower, the effect of delayed addition of histidine.

in consumption of solution A and of the basal ration during period 3, their total average weight gain for the 10 days was only 25 Gm. per rat.

Essentially similar results were obtained with respect to variations of the intake of phenylalanine and methionine with the exception that there was no set-back with methionine given in minimal amounts during period 2, and little if any with phenylalanine. Evidently these "minima" are close to those previously determined by us for repletion of the protein-depleted rat.

In chart 3 is shown a comparison of performance in the presence and the absence of histidine in solution A. Note especially that during period 2, when histidine was absent, consumption of the amino acid solution declined approximately 87 per cent in the four days, whereas the fall-off in consumption of the basal ration was only 15 per cent. Nevertheless, the animal in the four days lost more weight than he had gained during the first three days.

Experiment 3: Threonine, Lysine, Valine, Isoleucine, Leucine and Arginine.—For determining the effects of variations of amino acid composition with respect to the remaining essential amino acids, solution B was used. The results are given in table 4. Here, also, it is seen that in

TABLE 4.—Influence of Variation in Intake of Threonine, Lysine, Valine, Isoleucine, Leucine and Arginine on Consumption of Food, Drink and Weight over a Period of Ten Days

Amino Acid	Daily Amount in 25 Ml., Mg.	Rats	Period 1 (3 Days)			Period 2 (4 Days)			Period 3 (3 Days)			Weight Gain (Total), Gm.
			Liquid Consumed, %	Ration Eaten, %	Weight Gain, Gm.	Liquid Consumed, %	Ration Eaten, %	Weight Gain or Loss, Gm.	Liquid Consumed, %	Ration Eaten, %	Weight Gain, Gm.	
Threonine.....	132	5	96	96	22	100	100	22	100	90	7	51 ± 1.73
Threonine.....	80	5	96	95	24	100	90	13	100	90	11	48 ± 2.0
Threonine.....	0	5	99	95	24	25	92	-25	84	80	21	29 ± 3.6
Lysine.....	103.2	5	94	96	26	100	100	14	100	100	13	55 ± 1.63
Lysine.....	70.5	5	100	96	30	100	90	19	100	100	13	54 ± 1.74
Lysine.....	0	5	100	98	20	43	86	-15	51	95	15	31 ± 3.5
Valine.....	102.3	5	99	91	22	100	100	10	100	90	12	53 ± 2.23
Valine.....	102.0	5	98	90	22	100	97	14	100	99	13	49 ± 1.00
Valine.....	0	5	95	87	25	25	75	-17				
See chart 5 for period 3.												
Isoleucine.....	100.0	4	96	99	10	100	90	20	100	100	10	49 ± 1.26
Isoleucine.....	122.0	5	92	89	18	98	92	15	100	99	11	44 ± 2.34
Isoleucine.....	0	5	94	88	23	19	66	-25	93	90	29	35 ± 4.47
Leucine.....	104.8	5	97	86	22	100	98	21	100	95	13	56 ± 2.23
Leucine.....	72.5	5	94	91	23	93	98	-1	90	96	18	40 ± 2.64
Leucine.....	0	5	92	86	24	19	53	-30	100	89	28	32 ± 1.58
Arginine HCl.....	65.2	5	97	93	25	95	99	23	95	99	11	59 ± 1.58

* In period 1 all lysine groups received one extra offering of solution B.

each instance during period 2 omission of threonine, lysine, valine, isoleucine or leucine led to a sharply lowered acceptance of the solution and a concomitant decline in consumption of the basal ration. In each instance, also, there was an obvious loss of weight. Only in the case of leucine was there a definite deterioration in nutritive performance when minimal amounts of amino acid were present in solution B. With isoleucine, valine, lysine and threonine it is apparent that the "minima" were close to the amounts required. The time interval of four days, however, is too short to determine the effects precisely, but in any case the results reaffirm the approximate validity of the "minima" as previously established by us. It is of interest that the absence of arginine from solution B had no adverse effect and that during the seven days the weight recovery was as good or better than when arginine was present.

The effects of threonine deficiency are shown in chart 4 where it can be seen that the intake of solution B lacking threonine was reduced approximately one half on the first day following its removal, whereas consumption of the basal ration fell off only about one tenth. After the threonine-deficient solution had been offered for five days, the offering of solution B for two days was accompanied by an immediately improved

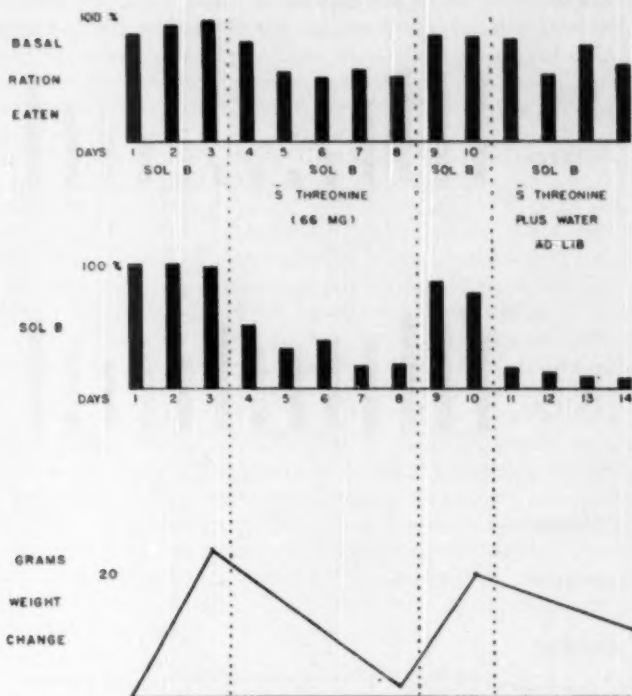


Chart 4.—Influence of threonine deficiency on protein-depleted rats' acceptance of fluid and food (average for five rats.)

acceptance of both the solution and the basal ration and by good weight gains. When threonine was again omitted for a final four days, but with water furnished ad libitum, acceptance of the deficient amino acid solution declined to 10 per cent or less, although food consumption averaged better than 50 per cent. Nonetheless the animals again began to lose weight.

Chart 5 illustrates the consequences of omitting valine from solution B. Here, too, on the first day acceptance of the amino acid solution declined about one third. Consumption of the basal ration, however, approximated 75 per cent, but with the animals losing weight. When water was given ad libitum for two days, consumption of the basal ration improved slightly for one day and then dropped back to the former level. Acceptance of the amino acid solution was poor during this interval. When solution B was again given, acceptance lagged for two days, along

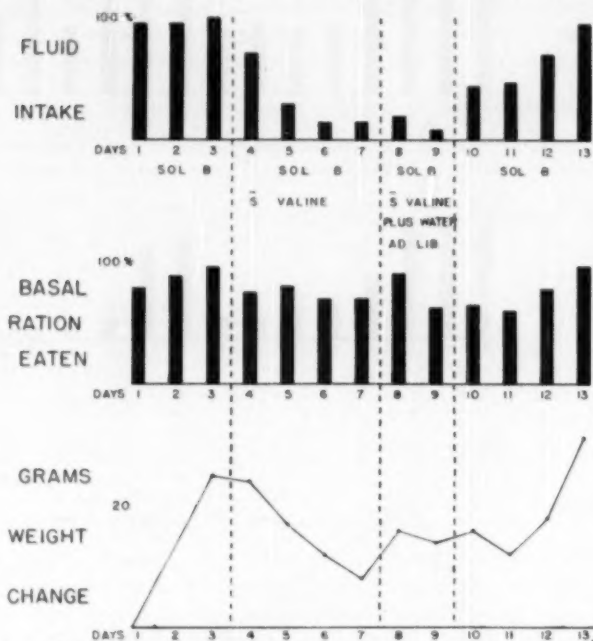


Chart 5.—Influence of valine deficiency on protein-depleted rats' acceptance of food and fluid (average for 5 rats.)

with consumption of the basal ration, and then returned to normal concomitant with a rapid weight recovery.

COMMENT

These experiments serve to reemphasize the fact that an essential amino acid deficiency in the protein-depleted rat leads speedily to a loss of weight even though the caloric intake remains adequate or almost so. Soon, however, the animal loses interest in both food and drink. One

might suppose that thirst would act as a stronger driving force than it seemingly does, but animals offered an amino acid solution devoid of a single essential amino acid may refuse to drink more than a few milliliters per day for at least a week, even when the temperature is above 90 F. It should be recalled, however, that the basal ration contains 16 per cent of water.

This method demonstrates the relative ease with which amino acid solutions of unknown composition, protein hydrolysates, for example, may be nutritively evaluated. Thus, when a solution under investigation is completely consumed, together with complete ingestion of the basal ration, weight recovery is rapid, thereby demonstrating an adequate content of all the essential amino acids necessary for effective tissue protein synthesis. If, however, one or more essential amino acids are present in limiting amounts, a slower weight recovery will point to the likelihood of an essential amino acid inadequacy. This may indicate either that there has been destruction of certain essential amino acids in the course of hydrolysis, with inadequate reconstitution, or it may indicate that there has been an interaction between an essential amino acid and some other dietary component, sugar, for example, leading to the formation of an amino acid-sugar complex which makes this particular amino acid unavailable to the action of intestinal enzymes. The method also offers a means of studying more adequately the consequences of mineral deficiencies. Thus, a particular salt can be omitted from the basal ration while at the same time the daily amino acid needs are satisfied by means of the amino acid solution. Similarly it may be possible by this method to get further information concerning such problems as amino acid imbalances, the influence of time on the relative availability of calories in relation to amino acid utilization, and the role of accessory growth factors in protein metabolism. We have already used the method to determine the comparative utilizability of some unnatural forms of essential amino acids.

SUMMARY AND CONCLUSIONS

A method has been described in which the repletion of protein-depleted adult male albino rats can be observed under conditions in which all dietary nitrogen is supplied as a solution of amino acids. Concomitantly the basal ration furnishes needed calories, vitamins, salts and roughage. By this method it has been possible to study the effects on tissue protein synthesis of an acute essential amino acid deficiency without any direct influence on acceptance of the basal ration through a possible modification of the latter's taste or odor. Nevertheless, if any one of nine indispensable amino acids was removed from the amino acid solution, its withdrawal was followed within twenty-four hours by a

significant decline in consumption of the solution and a drop in weight. Food consumption fell off more gradually.

Refusal to drink an amino acid solution lacking a particular essential amino acid is apparently not due primarily to an alteration in taste or odor, in view of the delayed refusal. Neither can it be attributed to an immediate caloric deficiency. Evidently the rapidly functioning mechanisms of protein metabolism require the simultaneous availability of all the essential amino acids for effective tissue protein synthesis, and in the presence of an acute essential amino acid deficiency, catabolism quickly ensues.

The usefulness of this method has been pointed out, particularly with reference to the nutritive evaluation of protein hydrolysates, as well as for the study of mineral inadequacies and other metabolic problems.

ENDOMETRIAL POLYPS AND HYPERPLASIA PRODUCED IN AN AGED MONKEY WITH ESTROGEN PLUS PROGESTERONE

ROBERT J. CROSSEN, M.D.

AND

VALENTINA SUNTZEFF, M.D.

ST. LOUIS

SPONTANEOUS malignant tumors are rare in subhuman primates. Carcinoma of the oral cavity¹ and of the prostate² and fibromyoma and papilloma of the cervix³ have been observed. The pertinent literature has been reviewed recently.⁴ One of the reasons for the paucity of such tumors is seen in the fact that the animals when kept in captivity do not live long enough for tumors to develop.

Prolonged administration of large doses of estrogen produced cervical metaplasia resembling early cancer⁵ and cystic glandular hyperplasia of the endometrium⁶ in young and adult monkeys and uterine bleeding in spayed rhesus monkeys.⁶ However, in spite of the vigorous proliferation, the endometrium gradually returned to normal when the estrogenic treatment was discontinued, and cancerous transformation could not be enforced.¹

MATERIAL AND METHODS

Old monkeys or monkeys of known age are difficult to secure. In October 1941 we obtained a female rhesus monkey whose age at the time of capture was unknown, but which had been in captivity for 2 years. In order to determine whether or not the monkey was in the menopause, we observed her daily, except on Sundays, for a period of six months. Twice the sexual skin showed a slight reddish tinge, once on Jan. 13, 1942 and again on April 3, 1942.

From the Barnard Free Skin and Cancer Hospital.

1. Pfeiffer, C. A., and Allen, E.: *Cancer Research* **8**:97, 1948.
2. Engle, E. T., and Stout, A. P.: *Am. J. Cancer* **39**:334, 1940.
3. Ratcliffe, H. L.: *Am. J. Cancer* **17**:116, 1933.
4. Overholser, M. D., and Allen, E.: *Proc. Soc. Exper. Biol. & Med.* **30**:1322, 1933.
5. (a) Engle, E. T., and Smith, P. E.: *Am. J. Anat.* **63**:349, 1938. (b) Hartman, C. G.; Geschickter, G. F., and Speert, H.: *Anat. Rec. (supp. 2)* **79**:31, 1941. (c) Engle, E. T.; Krakower, C., and Haagensen, C. D.: *Cancer Research* **3**:858, 1943.
6. Zuckerman, S.: *J. Endocrinol.* **2**:263, 1941.

On June 25, 1942, before the experiment was started, a curettage was done to determine the condition of the endometrium. With the animal under ether anesthesia, the vagina was dilated slowly by means of a small rectal dilator. The cervix was grasped with an Allis forceps, and the debris in the vagina was flushed out with sodium chloride solution. The cervix was then dilated with a small urethral dilator, and a curettage was done, using a dental curet. The tissue obtained was fixed in 4 per cent formaldehyde. Following the curettage, two 15 mg. pellets of estradiol benzoate were implanted in the subcutaneous tissue under the skin of the back. On August 31 another curettage was done; little tissue was obtained, and two 15 mg. pellets of estradiol benzoate were again implanted. On March 9, 1943 two more 10 mg. pellets of estradiol benzoate were implanted. On June 28 the curettage was repeated, and two 10 mg. pellets of estradiol benzoate were implanted. On Jan. 17, 1944 we started giving 5 mg. of progesterone (lutocyclin*)^{6a} (sublingual) twice daily for six days a week. The monkey has a tendency to hold food in its cheek pouch; hence it was thought that some of the sublingual medication would be absorbed parenterally and the rest would be swallowed and absorbed through the intestine. On February 19 another curettage was performed. On rectal examination the uterus seemed to be somewhat larger than at the start of the experiment. On July 25 the last tablet of progesterone was given, making a total of 1,630 mg. of the substance given over a period of 163 days. On September 30 an attempt at curettage was unsuccessful. During the vaginal dilation, a tear occurred through the sphincter ani, and this was immediately repaired. Two pellets of estradiol benzoate, 10 mg. each, were implanted. This final implantation made a total of 120 mg. of estradiol benzoate implanted over a period of a year and three months. On Feb. 9, 1945 a bloody vaginal discharge was noted. This was the first time that any vaginal bleeding was observed. On April 13 the monkey was sluggish and appeared to be ill. From May 5 on, the free bloody vaginal discharge became continuous, and during that month the monkey continued to fail in strength. The skin of the face was pale. On May 7 the monkey was unable to sit or stand up and was still bleeding from the vagina; so it was killed, and a necropsy was performed.

OBSERVATIONS

Gross Findings.—The uterus was enlarged and contained a large, dark-colored tumor, which could be seen partially protruding through the cervix. When the uterus was opened, this tumor was seen to be attached by a broad base to the lower end of the uterine cavity (fig. 1). The rest of the endometrium was thickened, and the surface was irregular. In some areas small polyps could be seen. The wall of the uterus was 2 cm. thick at its thickest portion, not including the endometrium, which varied from 2 to 4 mm. in height. The tumor rose 2.2 cm. above the endometrial surface. The vagina and cervix were also thickened. The ovaries were normal in size, and the right ovary had a hemorrhagic cyst on its surface.

Microscopic Observations.—The endometrial tissue from the first curettage, at the start of the experiment, showed resting glands of uniform size and shape, lined by low cuboidal epithelium (fig. 2). The tissue obtained at subsequent curettages did not consist of endometrium and hence was of no value. Most of it was composed of cervical glands and bits of squamous epithelium, evidently from the cervix.

^{6a} In this country the product is marketed under the name of lutocyclin* by Ciba Pharmaceutical Products Inc.

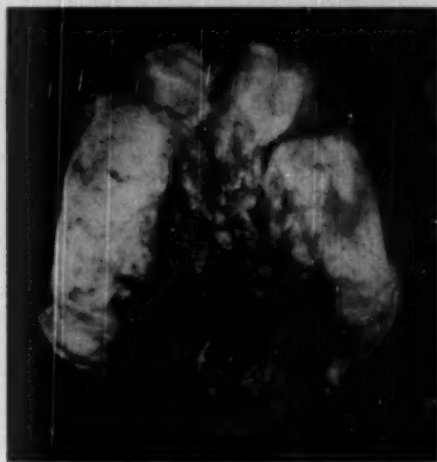


Fig. 1.—Opened uterus showing the large polyp protruding from the endometrium (approximately natural size).

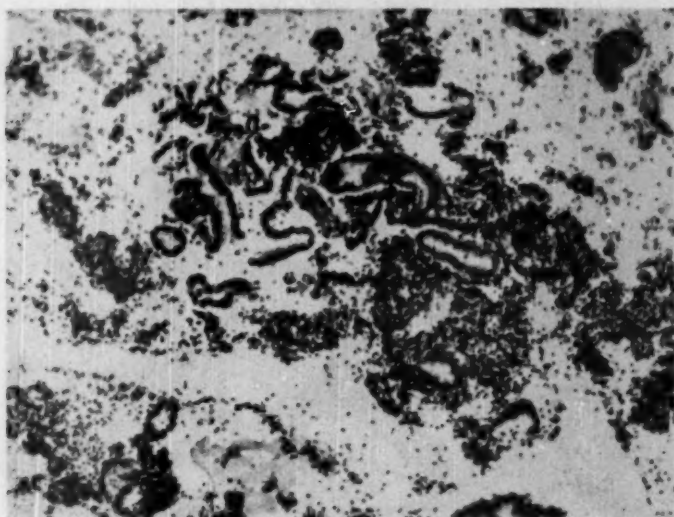


Fig. 2.—Photomicrograph of endometrial curettings before treatment (medium power magnification). The glands are resting.

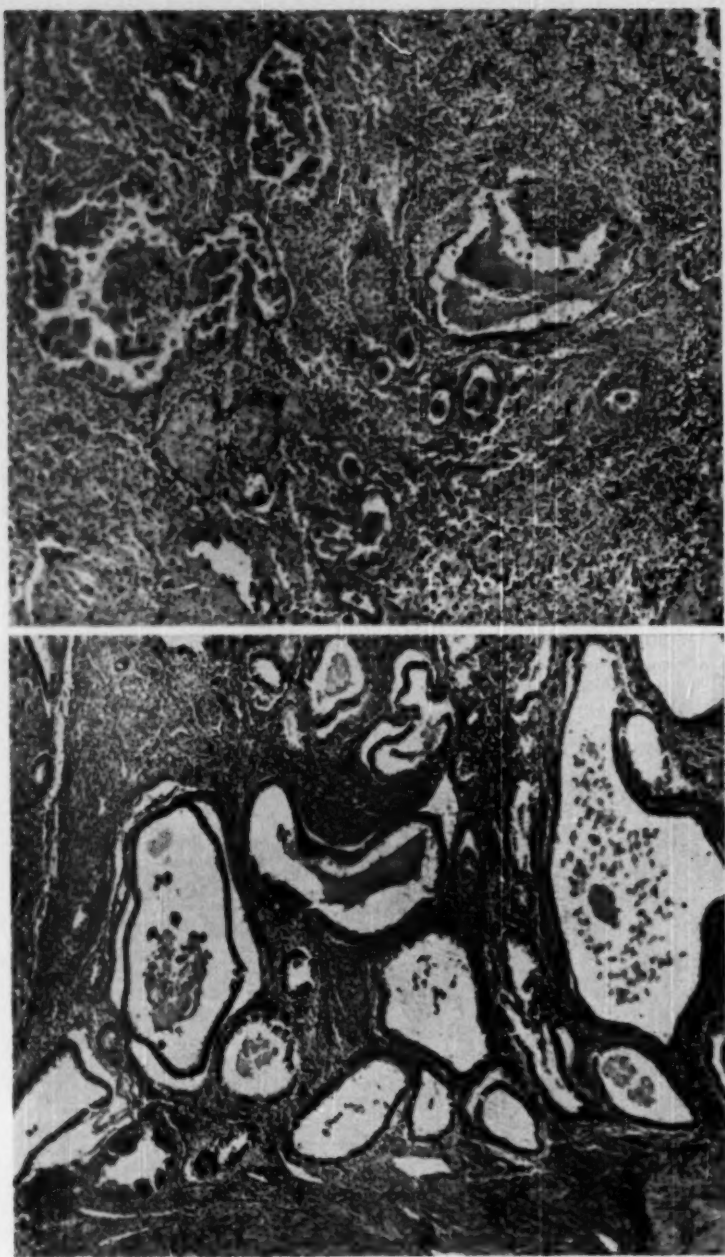


Fig. 3.—Upper part: Photomicrograph of an area from the adenomatous polyp (medium power magnification). Lower part: Photomicrograph of endometrial hyperplasia, Swiss cheese pattern (medium power magnification). In the upper right field can be seen a group of hypertrophied vessels.

The tumor proved to be a large polyp in which extensive hemorrhage had occurred. At the base of this polyp the endometrium showed an estrogenic (Swiss cheese) type of hyperplasia, and in this area there was little hemorrhage or degeneration. At the middle portion of this polyp there was an intense collection of red blood corpuscles, leukocytes and plasmacytes; the endometrial glands were disintegrating. At the tip of the polyp necrosis was advanced, and only fragments of glands were found (fig. 3, upper part).

The endometrium showed cystic hyperplasia (fig. 3, lower part). The glands varied in size and shape, and many were cystically dilated. They were lined by both cuboidal and low columnar epithelium, such as is seen in the interval stage. Some of the glands contained homogeneous debris. Throughout the endometrium the blood vessels were increased in number and thickness, containing a large amount of smooth muscle and a distinct media. These changes were recently described by Schwarz and Sherman⁷ as a component of endometrial hyperplasia in women and as seen in experimentally produced endometrial hyperplasia of rabbits.

The myometrium was markedly hypertrophic and likewise contained numerous thickened vessels.

The cervical glands were dilated, and there was secretion in the lumens of some of them. There was squamous metaplasia of the glandular and surface epithelium. The vaginal epithelium was notably thickened and disclosed superficial keratinization and shedding of cells into the vaginal canal. Below this was a broad middle layer, and in the basal layer the blunted papillae projected into the homogeneous tissue beneath them. The dark-staining basal layer resembled the eleidin layer seen in leukoplakia of the vulva in the human subject. There was a collagen-like substance in the underlying connective tissue stroma.

The uterine tube showed hypertrophy of the papillae, which were covered by epithelium consisting of tall columnar cells with basal nuclei. The vessels in the thickened musculature were larger than usual.

The ovary was covered by an inactive germinal epithelium, beneath which was a thick, fibrous tunica albuginea. The cortex contained numerous follicles in various stages of development. There were many light-staining areas, representing the remains of atretic follicles. The large hemorrhagic follicle noted in the gross description was lined by fragments of membrana granulosa, and there was old blood in the cavity. Another large follicle showed beginning degeneration.

The mammary glands contained greatly dilated ducts. The acinous tissue was much more abundant than that in a normal resting breast, but the epithelium disclosed no abnormal proliferation. Thus the findings were similar to those described by other investigators.⁸

COMMENT

The effects of estrogen and progesterone administration in our old female monkey were similar to those reported previously.⁹ In addition, our monkey had a large hemorrhagic endometrial polyp. Attention is also drawn to the extensive hypertrophy and hyperplasia of the blood vessels

7. Schwarz, O., and Sherman, A.: *Am. J. Obst. & Gynec.* **50**:1330, 1950.

8. Pfeiffer and Allen.³ Overholser and Allen.⁴

9. Pfeiffer and Allen.³ Overholser and Allen.⁴ Engle and Smith.^{5a} Hartman, Geschickter and Speert.^{5b} Engle, Krakower and Haagensen.^{5c} Zuckerman.⁶

in the wall of the uterus, in the endometrium and in the polyp. This finding in the vessels usually accompanies endometrial hyperplasia in woman, in the rabbit and also in the monkey.⁷

We have, for many years, been interested in the importance of age as a factor in the development of endometrial carcinoma.¹⁰ In the human subject it is evidently an important factor, for endometrial carcinoma occurs most frequently past middle life. Whether this high incidence of carcinoma with advancing age is due to a lessened ability of the tissues to control growth or whether it is due to stimulation by endogenous substances which may act more frequently and for longer periods, cannot be decided; it is probably due to a combination of both factors.

Endometrial carcinoma is four times as frequent in women who menstruate past the age of 50 years as it is in women having the menopause at the usual age.¹¹ These findings suggest that the action of the endogenous estrogen on an aging endometrium may be the factor resulting in cancerous change.

The effect of long-continued estrogen administration in old mice of inbred strains was studied by Crossen and Loeb.¹⁰ In one old mouse endometrial changes were produced resembling those seen in adenocarcinoma (adenoma malignum) of the human endometrium. It was concluded that the increased incidence of cancer with advancing age is largely due to the cumulative effect of repeated growth stimuli. As far as growth and cancerous processes are concerned the tissues of older and of younger organisms seem to differ only quantitatively in their reactions; the mode of reaction seems to be similar. The addition of exogenous to previously active endogenous stimuli may intensify certain growth processes initiated by the latter or may induce other growth processes.

SUMMARY

An aged female monkey was given 120 mg. of estradiol benzoate by implantation plus 1,630 mg. of progesterone (lutocyclin*) by mouth over a period of three years. In addition to pronounced cystic hyperplasia of the endometrium, a large hemorrhagic endometrial polyp was produced. Hypertrophy and hyperplasia of the blood vessels were present in the polyp, the endometrium and the muscle wall. The role of endogenous and exogenous estrogens acting on aging tissues is discussed.

10. Crossen, R. J., and Loeb, L.: *Arch. Path.* **37**:202, 1944.

11. Crossen, R. J., and Hobbs, J. E.: *J. Missouri M. A.* **32**:361, 1935.

PITUITARY, ADRENAL AND THYROID IN CYCLOPIA

HENRY W. EDMONDS, M.D.

Chief of Pathology and Anatomy, Medical Museum, Armed Forces Institute of Pathology
WASHINGTON, D. C.

CYCLOPIA is a distinctive and uncommon congenital malformation in man in which there is partial or complete fusion of the eyes. The structural abnormalities of the eyes and their adnexa and the associated changes in the skull and in the brain (the characteristic malformation designated as arhinencephaly) have long been adequately described.¹ As part of the regional deficit of structures that normally develop in the midline at and below the anterior end of the neural plate, cyclopic fetuses may lack a pituitary. It may then be asked whether the absence of the pituitary affects the development of other endocrine glands. The answer to this question is not to be found in the literature. It is therefore the purpose of the present paper to describe the thyroid and the adrenals as observed in three cyclopic fetuses lacking a pituitary and in two cyclopic fetuses having a pituitary.

In anencephaly, a relatively common malformation in man, it has been known, at least since the time of Ballantyne,² that the pituitary is generally smaller than usual as a result of regional malformation of the structures at the base of the brain. While it was once thought that the pituitary might be absent in anencephaly, the careful studies of Covell,³ Václav,⁴ Angevine,⁵ and Ch'in⁶ have established with reasonable certainty that the anterior lobe of the pituitary is present in all cases of anencephaly, though it is distinctly smaller than in the normal fetus. It is agreed by the authors just cited that in anencephaly the adrenals are

1. Schwalbe, E., and Josephy, H.: *Die Cyclopie*, in Schwalbe, E.: *Die Morphologie der Missbildungen des Menschen und der Tiere*, Jena, Gustav Fischer, 1913, pt. 2, chap. 5.

2. Ballantyne, J. W.: *Manual of Antenatal Pathology and Hygiene*, Edinburgh, William Green & Sons, 1904, vol. 2, *The Embryo*.

3. Covell, S. P.: *Am. J. Path.* **3**:17, 1927.

4. Václav, J.: *Sborn. lék.* **28**:399, 1927.

5. Angevine, D. M.: *Arch. Path.* **26**:507, 1938.

6. Ch'in, K. Y.: *Chinese M. J.*, 1938, supp. 2, p. 63.

characteristically hypoplastic, with a cortex patterned after the adult arrangement of layers, lacking the fetal or "X" zone, the involution of which is so prominent a feature of the normal neonatal adrenal. In regard to the state of the other endocrine glands in anencephaly, opinions have been less concurrent. According to Ch'in, the thymus is characterized by hyperplasia and the thyroid by moderate enlargement (from distention of the acini with accumulated colloid), while the gonads are hypoplastic (owing to marked reduction in the number of the interstitial cells). According to Václav, the development of the thyroid is arrested, the acini being filled with cells and not with colloid. Václav described the gonads as retarded and the thymus as variable and without distinctive characteristics. Angevine found no microscopic changes in thyroid, thymus or gonads. It would appear that further study of these endocrine organs in anencephalic fetuses is indicated.

In cyclopia the malformation of the structures at the base of the brain creates, in regard to the pituitary, a situation partly analogous to that existing in anencephaly. It is noteworthy that there have been no correlative studies of the endocrine glands in cyclopia as there have been in anencephaly. It has been recognized that the pituitary may be absent. In 63 cases reported in papers located primarily through the Index Catalogue, Fourth Series,⁷ the presence or the absence of the pituitary was specified only eleven times. It was reported as absent in six cases. In only two of the 63 cases reported was mention made of the state of the thyroid or the adrenals. Rothschild⁸ described a cyclopic fetus lacking both pituitary and adrenals. Ozawa⁹ noted smallness of thyroid, adrenals and testes in a cyclopic fetus without pituitary.

Because of the rarity and striking appearance of cyclopic specimens, many have been preserved for museum display, without adequate internal dissection. Too, it is apparent from many of the reports in the literature that attention has been focused on the morphologic changes in the brain and the ocular structures to the exclusion of the remainder of the body.

Whole gross specimens of five cyclopic fetuses are preserved in the Medical Museum of the Armed Forces Institute of Pathology. The essential features of these specimens are as follows:

CASES OF CYCLOPIA WITH ABSENCE OF PITUITARY

CASE 1 (A. F. I. P. Accession 295238).—A female cyclopic fetus, sitting height 17.5 cm. and foot length 4.5 cm., has a tiny nasal proboscis situated above a single

7. Index-Catalogue of the Library of the Surgeon General's Office, Washington, D. C., Government Printing Office, 1938, series 4, vol. 3, pp. 1038-1039.

8. Rothschild, P.: Beitr. z. path. Anat. u. z. allg. Path. **73**:65, 1925.

9. Ozawa, M.: Japan. J. M. Sc., V, Path. **4**:189, 1939.

eye that has a single pupil. Internally, neither pituitary nor optic nerve is present. The gestational age is 22 weeks, estimated by the tables of Streeter.¹⁰

CASE 2 (A. F. I. P. Accession 295237).—A female cyclopic fetus, sitting height 31.5 cm. and foot length 7 cm., has a well developed nasal proboscis situated above a single eye that has two pupils. Internally, both optic nerve and pituitary are absent. There is malformation of the heart and great vessels, involving transposition of the arterial trunks and retention of the right aortic arch in lieu of the left. The liver and the spleen are laterally inverted, and the bowels lie in a position of inverse rotation. The estimated gestational age is 35 weeks.

CASE 3 (A. F. I. P. Accession 292104).—A male cyclopic fetus, sitting height 32.5 cm. and foot length 7 cm., has a well developed nasal proboscis situated above a single eye that has two pupils. Internally there is neither optic nerve nor pituitary. The estimated gestational age is 35 weeks.

CASES OF CYCLOPIA WITH PRESENCE OF PITUITARY

CASE 4 (A. F. I. P. Accession 280256).—A female cyclopic fetus, sitting height 22.5 cm. and foot length 5.3 cm., has two small nasal proboscises situated one above and one below a single eye that has two pupils. Synotia is present, accompanied with micrognathia. Internally a single optic nerve and a pituitary are found. There are persistent right and left superior caval veins. The arterial trunks of the heart are transposed, with a right aortic arch. Stomach, liver and (duplicated) spleen are laterally inverted. The bowels are in a position of nonrotation. The estimated gestational age is 26 weeks.

CASE 5 (A. F. I. P. Accession 295239).—A male cyclopic fetus, sitting height 33 cm. and foot length 7.2 cm., has a single eye with a single pupil. No nasal proboscis is present. Internally an optic nerve and a pituitary are found. The estimated gestational age is 36 weeks.

In the dissections, no difficulty was experienced in deciding whether or not a pituitary was present. In the two cases in which the gland was located, it lay within a sella of normal form. In those cases in which a pituitary was lacking, there was likewise no sella, owing to marked reduction in the size of the sphenoid bone. The ease with which the pituitary may be found in cyclopic fetuses (when it is present) is in contrast with the difficulty frequently encountered in dissection of anencephalic fetuses, for in the latter the region of the sella is generally obscured by a thick carpet of hypertrophic congested meninges.

On removal of the endocrine glands in the dissection of the fetuses, tracings were made to represent, in silhouette, the maximum area of each gland. The outlines so obtained are reproduced in figure 1. It is apparent that the outlined areas for the thyroid gland are distinctly larger in the two cases in which a pituitary was present than in the three cases in which there was no pituitary. In the smaller thyroids the lateral lobes were separated from each other.

10. Streeter, G. L.: *Contrib. Embryol.* **11**:143, 1920.

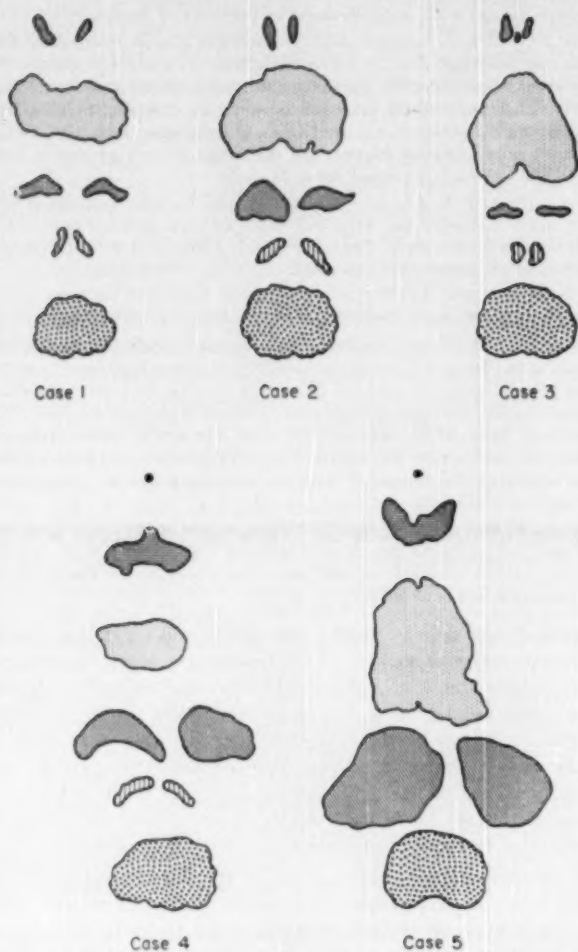


Fig. 1.—Silhouettes representing the maximum surface areas of the endocrine glands of five cyclopic fetuses, reduced to half natural size. The pituitary (present in cases 4 and 5 only) is drawn in black, the thyroid in horizontal ruling, the thymus in fine stippling, the adrenals in diagonal ruling, the gonads in vertical ruling and the kidney in coarse stippling.

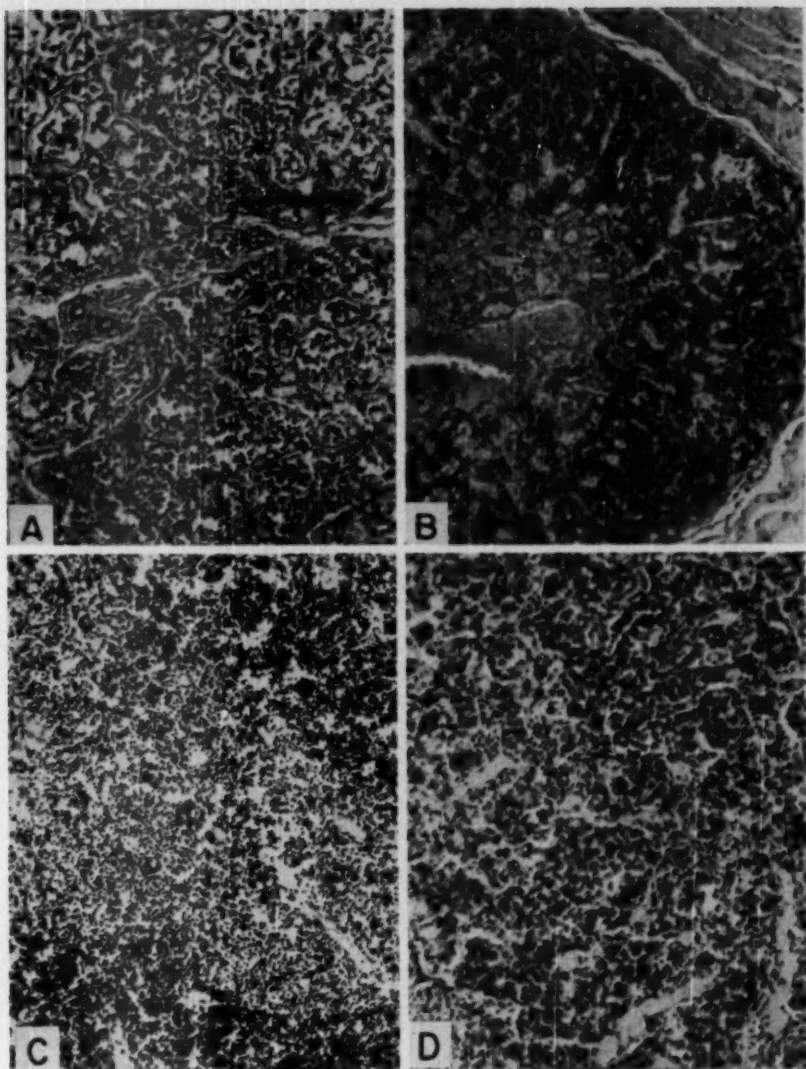


Fig. 2 (case 1).—Photomicrographs of (A) thyroid, (B) adrenal, (C) thymus and (D) ovary from a cyclopic fetus that lacked a pituitary (estimated gestational age, 22 weeks). The adrenal cortex is narrow, without a "fetal" zone. Hematoxylin and eosin stain; $\times 100$. Armed Forces Institute of Pathology Acc. 295238.

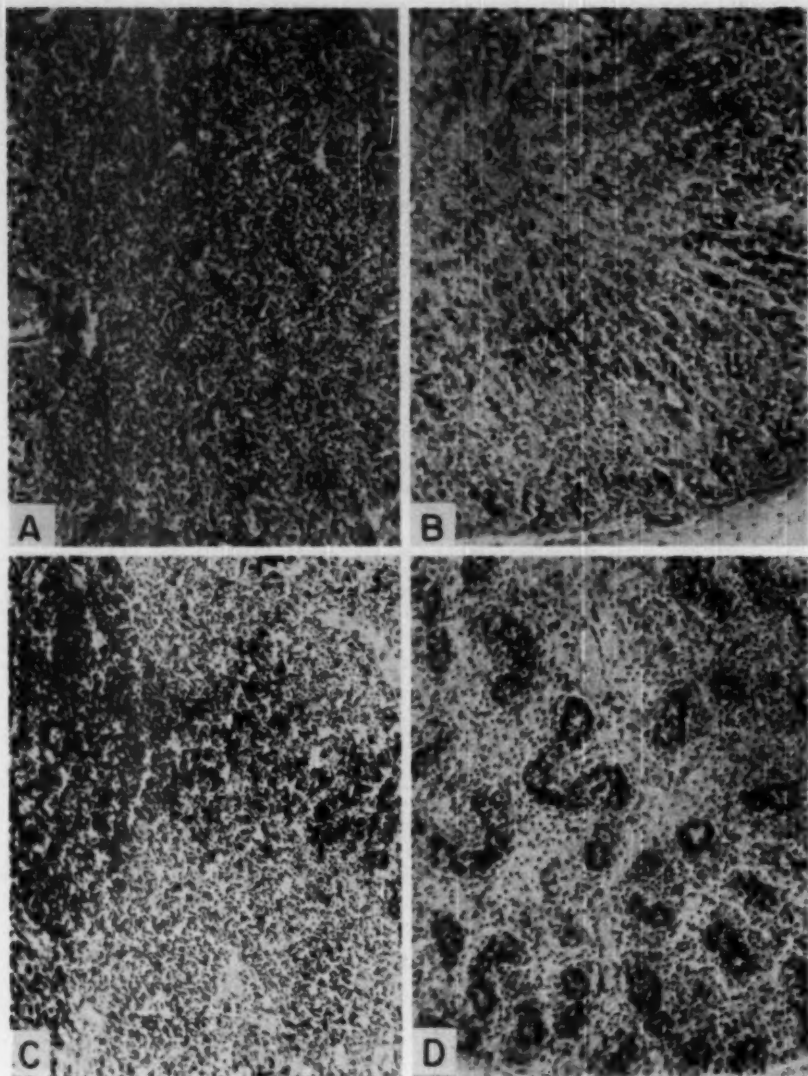


Fig. 3 (case 3).—Photomicrographs of (A) thyroid, (B) adrenal, (C) thymus and (D) testis from a cyclopic fetus that lacked a pituitary (estimated gestational age, 35 weeks). The adrenal cortex is narrow, without a "fetal" zone. Hematoxylin and eosin stain; $\times 100$. Armed Forces Institute of Pathology Acc. 292104.

It will be noted that the adrenals were relatively small in the three cases in which a pituitary was lacking and relatively large (normal for the age) in those cases in which a pituitary was present.

There is greater variability in the size of the thymus. A distinction between the thymus in those cases with pituitary and those without pituitary is not apparent in the tracings. Similarly, no significant differences are apparent in regard to the gonads. A tracing of one of the kidneys is added in each case to show the size of a representative non-endocrine organ.

MICROSCOPIC OBSERVATIONS

In cases 1 and 3 of the present study, sections of the adrenals show a narrow cortex without the broad fetal cortical zone which is so characteristic of the adrenal of this age group. Sections of thyroid, thymus and gonads (ovary in case 1 and testis in case 3) show no significant deviation as compared with the normal. The sections are pictured in the photomicrographs of figures 2 and 3.

Cases 2, 4 and 5 of the present study are represented by older specimens in which preservation of tissue detail is not adequate for satisfactory histologic examination. In cases 4 and 5, however, serial sections of the pituitary serve to confirm the gross identification of this gland and to demonstrate that it is comprised entirely of anterior lobe without neural components.

COMMENT

The gross and microscopic changes described here as observed in cyclopic fetuses lacking a pituitary are comparable to those described as obtaining in anencephaly. In regard to the mechanism of the alterations observed in the endocrine glands in anencephaly, both Ch'in and Angevine take the association of selective cortical hypoplasia of the adrenals to stand in obvious anatomic relationship with the quantitative deficiency in the anterior hypophysis. Angevine, however, qualifies his acceptance of such a relationship in the statement "The functional relationship of the two endocrine organs is defined with difficulty because anencephalic fetuses are continually under the influence of hypophyseal hormones of the mother."

The degree to which the placenta can be permeated by the hormones of the anterior lobe of the pituitary has been incompletely assessed. According to Loeb¹¹:

11. Loeb, L.: *The Biological Basis of Individuality*, Springfield, Ill., Charles C Thomas, Publisher, 1945, p. 167.

. . . The union between child and mother in the uterus, by means of the placenta, may be considered as a modified state of parabiosis, in which both organisms lead largely an independent life and in which both carry on their own metabolism, but in which to a certain degree an exchange of substances may take place through the placenta.

Aron¹² demonstrated that the placenta was nonpermeable to the hormones of the anterior lobe of the pituitary, using guinea pigs as experimental animals and the fetal thyroid as indicator. Cattaneo¹³ demonstrated that the placenta was permeable to posterior pituitary hormones, to epinephrine and to choline, using sheep as experimental animals and fetal blood pressure as indicator.

The findings in both cyclopia and anencephaly in regard to a reduction of the adrenal cortex associated with absence or quantitative diminution of the anterior lobe of the pituitary lend support to the belief that the placenta is not permeable to the hormones of the anterior lobe of the pituitary. An alternative hypothesis—that in cyclopia and in anencephaly there exists an insufficiency of anterior pituitary principles in the mother—may be considered beyond possibility since cyclopia and anencephaly have each been observed affecting only one member of twins as well as only one component of a double monster.

The stimulating effect exerted by the pituitary on the thyroid has been well known since the experiments of Loeb and his associates¹⁴ leading to the demonstration of the thyreotropic hormone of the pituitary, and needs no further comment.

An additional point of interest, following from the observations here reported, is the indication that the stimulating relationship of the anterior lobe of the pituitary to the adrenal cortex and thyroid is active in fetal life as well as in postnatal existence.

The pituitary-adrenal interrelationship may not be established in rats until several days after birth, as Jailer¹⁵ pointed out in a recent study showing that the neonatal rat adrenal responds to experimental injection of adrenocorticotrophic hormone and not to administration of epinephrine or to exposure to cold. Jailer suggested, however, that the rat pituitary is less mature at birth than is the human pituitary.

12. Aron, M.: *Strasbourg méd.* **90**:333, 1930.

13. Cattaneo, L.: *Arch. ital. de biol.* **90**:100, 1933-1934.

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SUMMARY

Examination of three cyclopic fetuses lacking a pituitary has shown reduction in the adrenal cortex comparable to that described in anencephaly, as well as smallness of the thyroid without histologic change. Examination of two cyclopic fetuses in which the anterior lobe of the pituitary was present has demonstrated normal development of the adrenals and the thyroid. These data support the hypotheses that the placenta is not permeable to the hormones of the anterior lobe of the pituitary and that the pituitary-adrenal and pituitary-thyroid interrelationships demonstrated in postnatal life are also active in the fetus.

EFFECT OF ROENTGEN RAYS ON THE TESTIS

Quantitative Histological Analysis Following Whole Body Exposure of Mice

ALLEN B. ESCHENBRENNER, M.D.

AND

ELIZA MILLER, A.B.

BETHESDA, MD.

SINCE 1903 there have been in the literature numerous reports concerning the histological changes observed in mammalian testes after a single roentgen ray exposure. The interpretation of the material with regard to the relative sensitivity of various components of the testes has been variable. The primary spermatocytes were considered the most sensitive by Bergonie and Tribondeau¹ and by Henshaw.² The spermatogonia were considered the most resistant by Wigoder³ and by Metz.⁴ As well summarized by Heller,⁵

Since the 1925 account of Schinz and Slotopolsky⁶ detailing and illustrating the changes produced by x-irradiation in the seminiferous tubules, however, the greater weight of opinion has held that the spermatogonia are first to be affected. This is the view of Regaud and Blanc⁷ and Lacassagne and Gricouroff⁸; it is the conclusion reached in the present study.

In an earlier publication^{9b} a report was made of quantitative histological analyses of the testes of mice observed at intervals of two months

From the United States Public Health Service, National Institutes of Health, National Cancer Institute.

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after the whole bodies of the mice had been exposed for 2 to 16 months to 8.8 r, 4.4 r or 1.1 r, respectively, of gamma radiation given in eight hours daily. It was found that despite obvious decrease of the total quantity of spermatogenic elements in the testes of chronically irradiated mice, the proportion of these cells in different stages of spermatogenesis was normal. It was tentatively concluded that chronic irradiation results in a retardation of the rate of multiplication of spermatogonia, cell death being minimal. To throw more light on the nature of the effect of irradiation on spermatogenic elements, a study has been made of the damage and recovery pattern following acute roentgen irradiation, and this forms the basis of the present report. Mice have been given whole body exposures of from 50 to 400 r, and serial studies have been made of the testes. A study has also been made, for comparison, of the damage and recovery following a second dose administered after the mice had recovered from the initial dose of radiation. For collateral interest, some data are included from preliminary experiments in studies on the damage and recovery pattern of the testes of mice exposed to radiations from a 10 mev betatron.

EXPERIMENTAL PROCEDURE

The mice were of the same strain as those used in earlier work,⁹ being LAF₁ hybrid males from colonies maintained at the National Cancer Institute. They were 3 months of age at the beginning of irradiation.

The low voltage roentgen irradiation of the whole body in doses of from 50 to 400 r was given under the following conditions: tube voltage, 170 kv. peak; tube current, 20.0 ma.; filter, 0.25 mm. copper and 0.5 mm. aluminum; focus to middle of mouse, distance 50 cm. The doses were administered at the rate of 64 r per minute, measured in air with a victoreen[®] thimble chamber r-meter.

The high voltage irradiation of the whole body was given under the following conditions¹⁰: The spectrum of the 10 mev Naval Ordnance Laboratory betatron determined by M. Wiener and T. Wang¹¹ at the National Bureau of Standards is 11.0 mev peak with a distribution in essential agreement with the theory of Heitler. The irradiations given were 165 r (33 r/min. for five minutes) and 300 r (37 r/min. for eight minutes) as measured in air with a victoreen[®] thimble chamber r-meter.

For serial study of the damage and recovery pattern following the different doses used for acute irradiation, 4 mice, together with 1 nonirradiated control, were killed at seven day intervals following the irradiation. The testes of the mice were dissected free of the epididymis and fat and weighed on a torsion balance. They were fixed in Zenker-formaldehyde solution for 4 to 5 hours, washed for 12 to 20 hours in running water and placed in 70 per cent ethyl alcohol for several days. Each was then cut into halves. These were dehydrated in ethyl alcohol and embedded in paraffin. The sections were cut 7 microns in thickness and stained with hematoxylin and eosin. Quantitative analyses of the testes were done by

10. In the betatron exposures, assistance was given by Mr. Donald O'Connor, of the Naval Ordnance Laboratory, and Dr. Theodore Wang, of the National Bureau of Standards.

11. Wang, P. K. S., and Wiener, M.: *Physiol. Rev.* **76**:1724, 1949.

means of Chalkley's method¹² as described in an earlier publication.¹⁰ This refers to the methodology and also to the criteria used for the identification of various cellular elements of the testes.

Studies on the effect of two successive acute irradiations were carried out under the following conditions: A group of mice, 3 months of age, were subjected to a 200 r whole body irradiation. At seven day intervals thereafter, four mice were killed and examined as described above. When it was observed that the weights of the testes of mice had returned to normal values 12 weeks after irradiation, the remaining mice were given a second 200 r whole body irradiation. At seven day intervals four mice were killed and examined as above.

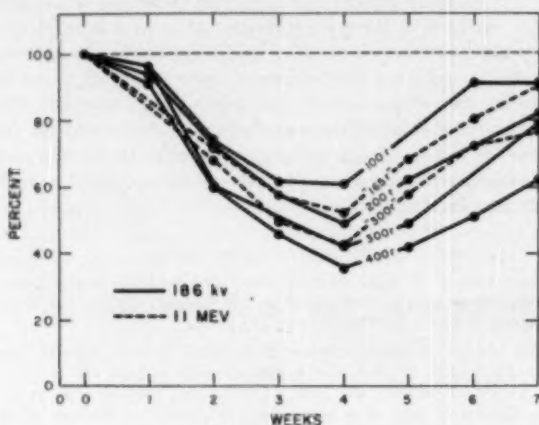


Fig. 1.—Effect of acute whole body roentgen irradiation as reflected by weights of testes. Each point on the curve is an average value for four mice. The testicular weights of the irradiated mice are expressed as per cent of weight of testes of non-irradiated mice of the same age.

RESULTS

The course of damage and recovery of the testes following a single whole body exposure to 50 r, 100 r, 200 r, 300 r or 400 r as reflected by the weight of the testes is shown in figure 1. It will be seen that the curves are qualitatively the same and differ with respect to the degree of loss of weight and also the time required for return of normal weight. What at first appears to be a remarkably long time required for an observation of a maximum effect, reflected in testicular weights, actually represents a masking of an immediate effect because of the selective sensitivity of a single stage of spermatogenesis, as brought out in the following histological observations.

12. Chalkley, H. W.: J. Nat. Cancer Inst. 4:47, 1943.

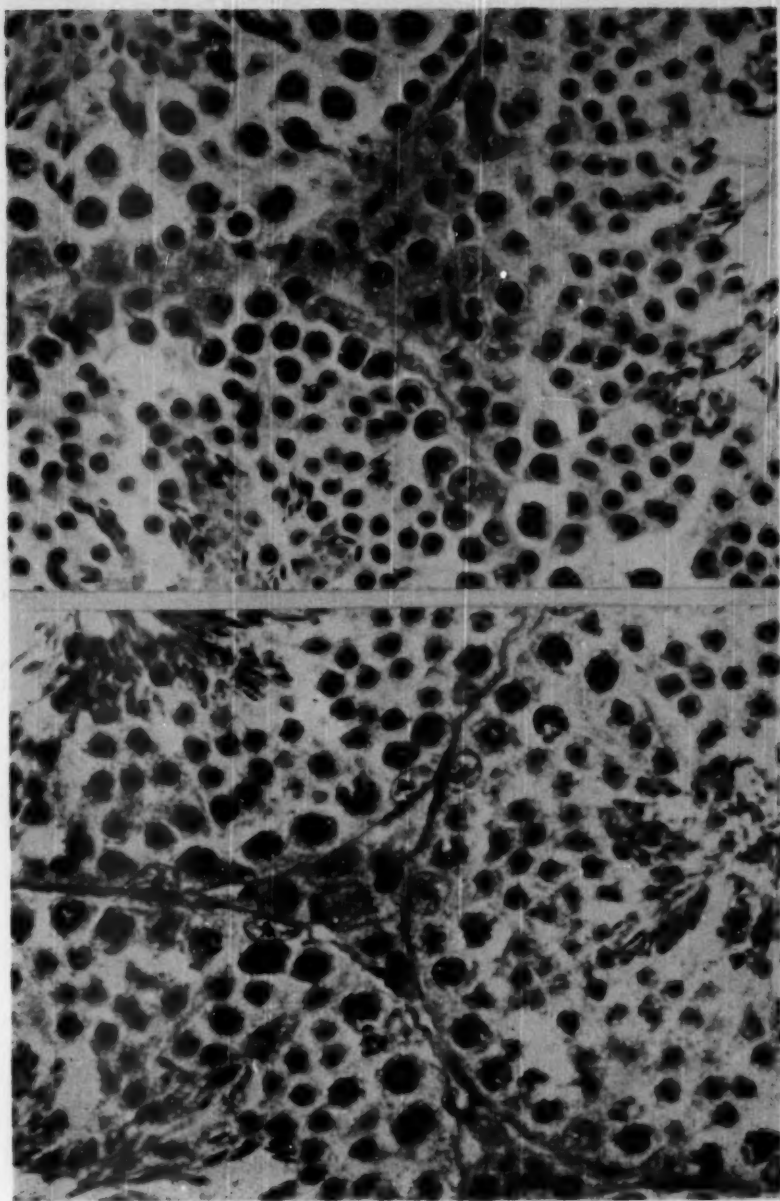


Fig. 2.—Upper part: Portions of three representative tubules of a normal testis showing spermatogonia, spermatocytes, spermatids and spermatozoa. $\times 700$.

Lower part: Portions of three representative seminiferous tubules of a testis one week after whole body exposure to 400 r of roentgen radiation. The population consists of spermatocytes, spermatids and spermatozoa. There is a conspicuous absence of spermatogonia. $\times 700$.

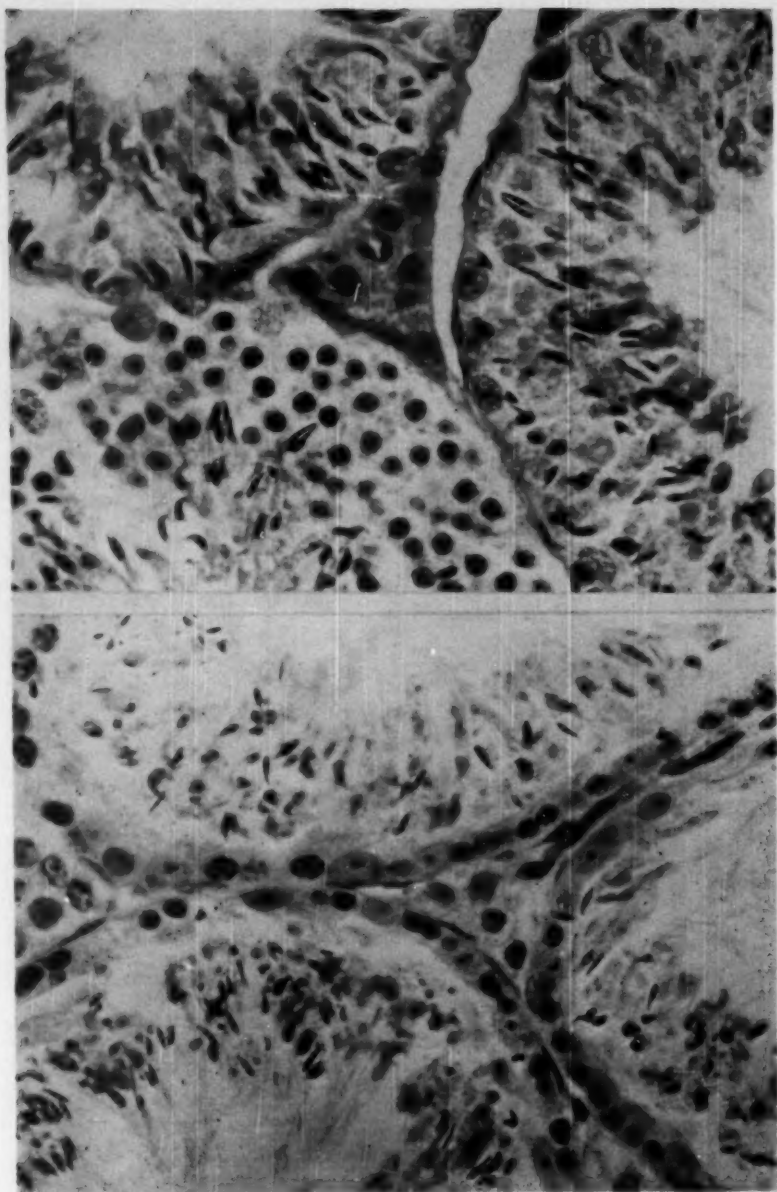


Fig. 3.—Upper part: Portions of three representative seminiferous tubules of a testis two weeks subsequent to whole body exposure to 400 r of roentgen radiation. The population consists only of spermatids and spermatozoa. There is a conspicuous absence of spermatogonia and of spermatocytes. $\times 700$.

Lower part: Portions of three representative seminiferous tubules of a testis three weeks after whole body exposure to 400 r of roentgen radiation. The population consists only of spermatogonia and spermatozoa. There is a conspicuous absence of spermatocytes and of spermatids. $\times 700$.

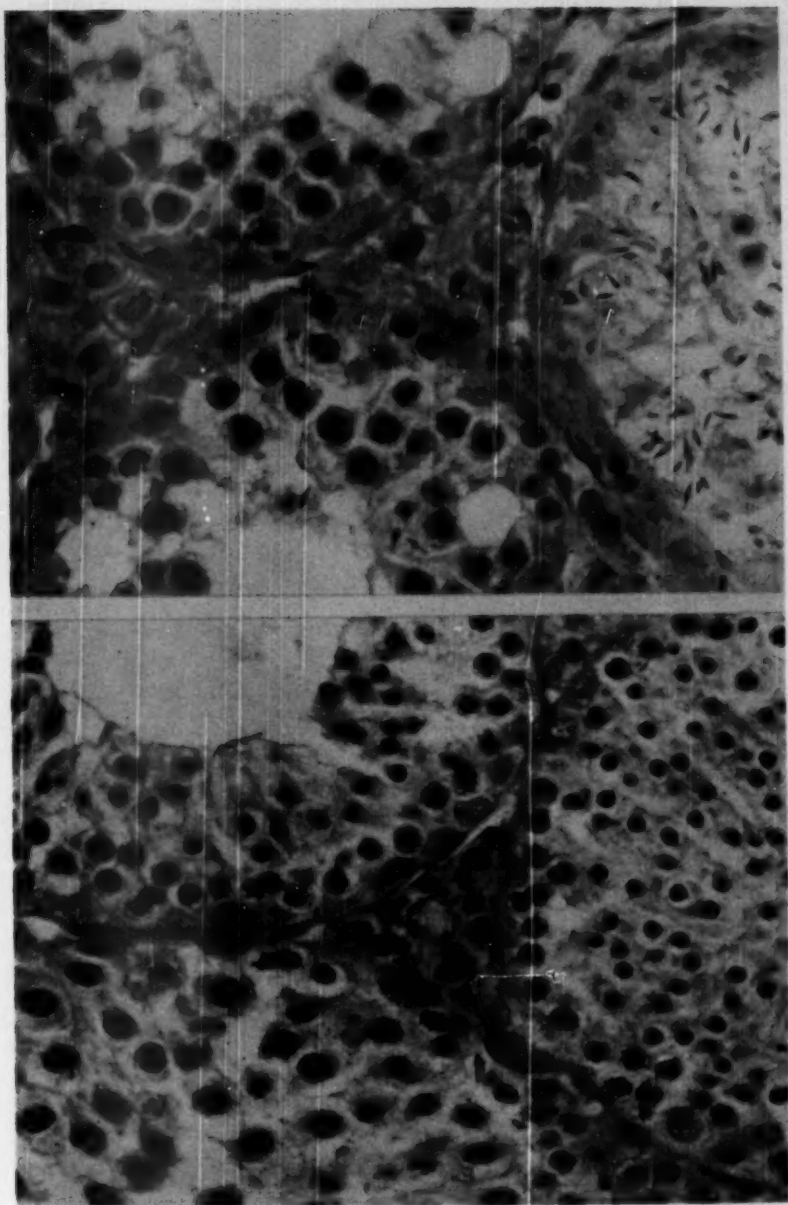


Fig. 4.—Upper part: Portions of three representative seminiferous tubules of a testis four weeks after whole body exposure to 400 r of roentgen radiation. The population consists of spermatogonia, spermatocytes and spermatozoa. There is an absence of spermatids. $\times 700$.

Lower part: Portions of three representative seminiferous tubules of a testis five weeks after whole body exposure to 400 r of roentgen radiation. The population consists of spermatogonia, spermatocytes and spermatids. Spermatozoa are absent. $\times 700$.

For comparison, a section of a normal testis is shown in figure 2, upper part. One week after irradiation the testes appeared normal except for a conspicuous absence of resting and of mitotic spermatogonia. A typical microscopic field seen one week after 400 r irradiation is shown in figure 2, lower part. Two weeks after the 400 r exposure there is an absence of spermatogonia and of spermatocytes and approximately normal numbers of spermatids and spermatozoa as shown in figure 3, upper part. Three weeks after the 400 r exposure the spermatogonia have increased markedly in numbers, and the only other spermatogenic

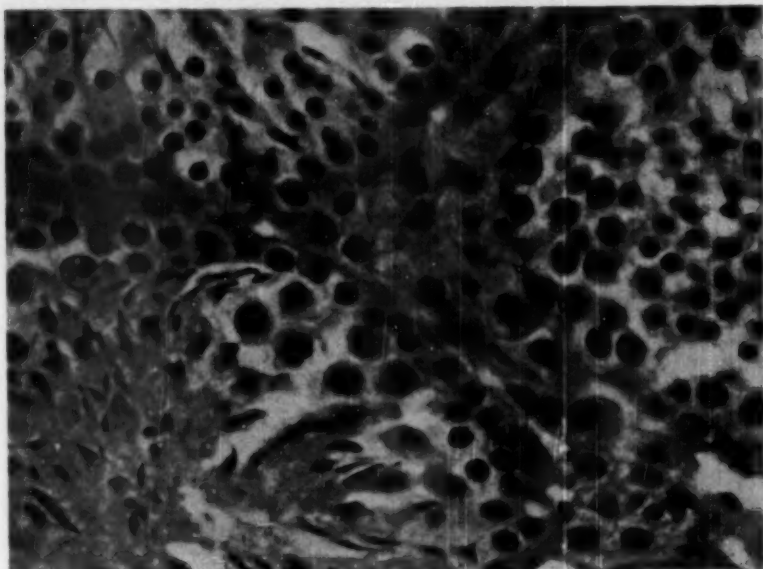


Fig. 5.—Portions of three representative seminiferous tubules of a testis six weeks subsequent to whole body exposure to 400 r of roentgen radiation. The population, with regard to various stages of spermatogenesis, has returned to normal, although the total numbers are probably less than normal in view of the observation that the weight of the testis was much less than normal. $\times 700$.

elements remaining in the tubules are mature spermatozoa as shown in the lower part of figure 3. Four weeks after exposure one finds spermatogonia, spermatocytes and a very few spermatozoa, as shown in figure 4, upper part. Five weeks after exposure one finds all spermatogenic elements except mature spermatozoa (fig. 4, lower part). Six weeks after irradiation the tubules of the testes contained all spermatogenic elements from spermatogonia to mature spermatozoa (fig. 5),

although the population is not yet normal in numbers as shown in the testicular weight curves (fig. 1). Thus, subsequent to irradiation, the spermatogonia, which are the source of other spermatogenic elements, are depleted, but the elements in any other given stage of spermatogenesis continue what appears to be a normal maturation process. After recovery from the effects of irradiation the spermatogonia multiply and become a source of successive stages of spermatogenesis. Although at three weeks after irradiation the spermatogonia have recovered and their total number probably exceeds that found in a normal testis, a further decrease of testicular weight occurs at the fourth week in the instance of animals exposed to 200 r, 300 r or 400 r, as shown in figure 1. This does not represent a discrepancy, as will be brought out in the comment,

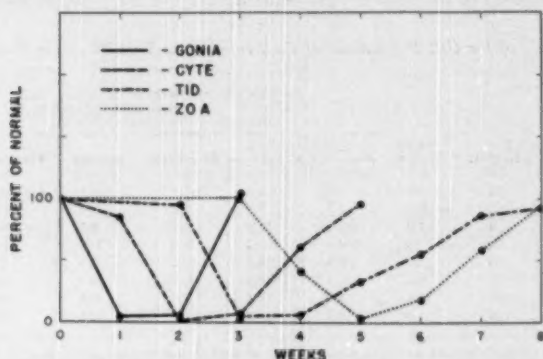


Fig. 6.—Cell population of the seminiferous tubules of the testes of mice following whole body exposure to 400 r. The proportions of the cells in different stages of spermatogenesis are shown as percentages of the values for cells in such stages in nonirradiated testes. Each point on the curve represents an average value for four mice.

if one considers the relative sizes and numbers of cells in different stages of spermatogenesis.

In view of the controversy mentioned in the introduction, the cell populations of the seminiferous testes of mice exposed to 400 r were analyzed by means of Chalkley's method. The results of this analysis are shown graphically in figure 6 and are recorded in the table. It is important to consider that although the data do not represent cell numbers but represent areas, they are, however, a reflection of actual cell numbers.

While a nucleus which showed pyknosis or fragmentation was occasionally seen near the basement membrane of a tubule after irradiation, such nuclei were few.

The use of Chalkley's method to obtain quantitative data with regard to cell populations of the seminiferous tubules gives ratios of total areas of nuclei of a given stage of spermatogenesis, expressed in terms of per cent of such values found in normal testes.²⁶ This is a reflection of cell numbers with the exception of one stage of the recovery process. It was quite noticeable that during the period preceding the reoccurrence of mitosis of spermatogonia, some nuclei of resting spermatogonia were larger than those seen in a normal testis. Such large nuclei of spermatogonia were seen during the period between 14 and 21 days. At this time, therefore, the total area of nuclei of spermatogonia is not as truly a reflection of cell numbers as it is at other times.

While quantitative histological analyses of the testes of mice exposed to 50 r have not yet been completed, there is to us no question that they

*Analysis of the Cell Populations of the Seminiferous Tubules of the Testes**

Weeks After Ir- radiation	Body Weight, Gm.	Testes Weight, Mg.	Spermatogenic Elements: Relative Total Area of Random Samples of Nuclear Material in Histologic Sections				
			Spermatogonia		Spermato- cytes, %	Sperma- tids, %	Spermatozoa Heads, %
			Resting, %	Mitotic, %			
1	31	308	2.3	0	65.1	101.2	113.43
2	30	148	4.7	3.0	2.8	96.8	107.9
3	34	108	165.0	27.3	5.3	2.5	132.5
4	34	79	217.2	225.2	30.4	11.3	39.5
5	30	90	186.5	162.1	94.8	32.8	5.0
6	32	118	167.0	149.0	94.7	29.8	18.4
7	29	141	169.0	66.2	109.0	90.4	56.7
8	31	158	100.0	106.0	96.7	92.8	95.2

* The results given were obtained by applying Chalkley's method to an analysis of the cell populations of the seminiferous tubules of the testes of mice after these had received whole body irradiation with 400 r of roentgen rays. Each value represents an average for four mice, expressed as per cent of normal for a given stage of spermatogenesis.

reveal evidence of only a partial inhibition of mitoses of spermatogonia in contrast with a two week complete inhibition seen in testes of mice exposed to 400 r. The degree of retardation of mitoses cannot be established until further quantitative histological analyses are complete. The sections of testes, however, reveal spermatogonia in mitosis throughout the postirradiation period. The assumption of the occurrence of retardation is based on the observed loss of weight of the testes, as will be brought out in the comment.

The damage and recovery pattern of the mouse testes as reflected in testicular weights resulting from a second acute roentgen exposure after the mouse had recovered from an initial exposure is shown in figure 7. In this figure one sees that the pattern of loss and recovery of weight is the same for a second acute exposure after the testes have returned to normal weight subsequent to an initial exposure. It is assumed, of

course, that the curve following the first exposure is a reflection of what had also taken place in the mice which were killed at weekly intervals to establish the curve for response to a second exposure.

The course of damage and recovery of the testes of mice exposed to the 11.4 mev betatron is also shown in figure 1. Cursory histological examination of sections of these testes shows no difference in response from that seen following exposure to 186 kv. roentgen rays. Quantitative histological analyses have not yet been carried out on these tissues. It will be seen that the curves obtained following exposure of mice to roentgen rays of the two different energies are the same. If one uses the loss and recovery of weight of the testis as an index, it can tentatively be assumed that one is dealing with the same biologic effect.

COMMENT

For sensitivity and reproducibility of results the testis is almost without parallel as a mammalian test organ for measuring the biologic

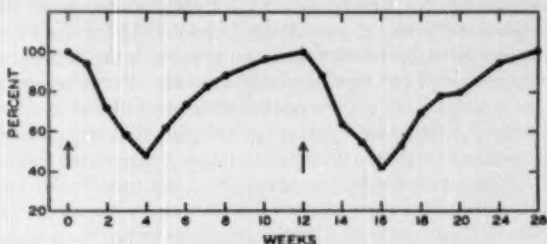


Fig. 7.—Effect of two successive acute whole body roentgen irradiations, as reflected by weights of testes. The time of irradiation is indicated by arrows. Each point on the curve is an average value for four mice. Testicular weights of irradiated mice are expressed as per cent of weight of testes of nonirradiated mice of the same age.

effect of ionizing radiations. The pattern of injury and recovery reflected in the weights of the testes is correlated with the dose administered. The minimum weights of the testes observed are dependent on dose. In addition to this, the time required to effect the minimum weights, as well as the time required for the weights to return to normal, is a function of dose. This was shown to be true for different doses of roentgen rays of 186 kv. Preliminary studies using the 10 mev roentgen rays indicate that it probably holds true also for roentgen rays in the multimillion volt range. A similar histological effect was also seen following chronic gamma irradiation.^{9b} The mouse testis will no doubt prove to be a valuable tool for subsequent studies on the relation between biologic effect and physical dose of ionizing radiations.

In a previously reported study^{9b} a quantitative histological analysis was made of the cell populations of the seminiferous tubules of the testes

of mice exposed to 8.8 r and to 4.4 r gamma radiation given in eight hours daily. This analysis showed that although the total numbers of cells in the seminiferous tubules markedly decreased as a result of chronic irradiation, there was a normal proportion of all stages of spermatogenesis. Since there was a near absence of degenerating forms, it was assumed that the effect of the chronic irradiation was largely one of retardation of the rate of multiplication of spermatogonia. The findings in the present experiments lend support to that tentative conclusion.

The histological observations in these experiments are of importance with regard to considerations of both the mechanism of the biologic effects of radiations and the normal histologic aspects of the spermatogenic elements. The near absence of morphologic evidence of cell death is striking. This is true even when the testes are reduced to 40 per cent of their normal weight on being exposed to 400 r. When it is considered that this decrease of weight is due entirely to decrease of quantity of spermatogenic elements, the interstitial tissue and supporting stroma remaining normal in total amounts,^{2a} the finding is even more striking than is at first apparent. A second significant histological observation is that the depopulation of the spermatogenic elements is due to the selective sensitivity of a single cell type, the spermatogonia. Furthermore, this is due not to destruction of spermatogonia, as mentioned, but to an inhibition of mitotic activity for a period of between two and three weeks following exposure to 400 r. While we cannot yet express the histological changes in the testes of mice exposed to 50 r in a quantitative manner, qualitative histological observations, together with data on weights of fresh testes, strongly point to a partial inhibition under these conditions of irradiation. The presumptive evidence for such partial inhibition is as follows: Loss of weight of testes of mice exposed to graded doses of radiation from 50 r to 400 r is due to decrease of total quantity of spermatogenic elements. In the testes of mice exposed to 400 r there is an absence of mitoses of spermatogonia seven and 14 days after irradiation. On the other hand, while the weights of testes of mice exposed to 50 r are decreasing, spermatogonia are seen in mitosis. Until quantitative histological data present evidence to the contrary, we can assume only that we are dealing here with a partial inhibition of mitoses of spermatogonia. If, now, this assumption proves to be correct, it indicates that in the case of spermatogonia the ionizing radiation causes a graded inhibition of mitoses in proportion to the dose administered. In other words, we probably are not dealing with an all or none effect.

Mitotic activity of spermatogonia in which they reproduce their own kind is considered to be similar to somatic mitosis. On the other hand, the process of maturation and division of the progeny of the spermatogonia is considered to be peculiar to germinal cells. In the normal testis

it is generally considered¹³ that during the maturation process in which spermatogonia develop into primary spermatocytes, one sees a heterotypic mitosis in which there is a simple allocation of the four sets of chromosomes to two daughter secondary spermatocytes. On a morphological basis the division of secondary spermatocytes each into spermatids (monads) is considered to be a homeotypic mitosis. This has been designated as homeotypic because each of the daughter spermatids receives one half of each of a pair of chromosomes from the spermatocyte as in the case of somatic mitoses. The fact that a retardation was not found in this cell division in the present experiments suggests that the morphologic similarity between this and somatic cell divisions is not a true reflection of the physiology of this cell division because of the difference in sensitivity to ionizing radiations.

It will be noted in figure 6 that the slopes of the curves representing decrease of each spermatogenic element are parallel. This suggests that the radiation administered had no discernible effect on the rate of spermatogenesis of various stages from spermatogonia to spermatozoa. The finding of a difference in slopes of the curves representing repopulation of various spermatogenic elements may reflect only a normal difference in the time required to proceed from one stage of spermatogenesis to the next, although this is speculation.

The observation that nuclei of resting spermatogonia are enlarged just before the return of mitotic activity of spermatogonia suggests that these nuclei are enlarging preparatory to going into mitosis but that for one reason or another they are unable to do so. It is natural to assume that a repair process is going on which involves metabolic systems that are essential to mitotic activity, although there is no morphological evidence to support this assumption. Similar large nuclei of spermatogonia were seen in the testes of mice chronically exposed to 4.4 r or to 8.8 r given in eight hours daily for 12 to 16 months.¹⁰ They were also occasionally seen in nonirradiated testes in the currently reported experiments.

The observation that the damage and recovery pattern of the testes reflected in the testicular weights after a second irradiation is the same as that following recovery from a first suggests, although it does not necessarily prove, that there is no residual metabolic damage in spermatogenic elements following recovery from sublethal doses of irradiation. The possibility that there is a variation in the sensitivity of different spermatogonia is unlikely because one would expect the less sensitive cells to reproduce their own kind in reconstituting the population of the testes. Such a reconstituted population might be expected to be less

13. Maximow, A. A., and Bloom, W.: *A Textbook of Histology*, ed. 4. Philadelphia, W. B. Saunders Company, 1942.

sensitive to irradiation, which does not seem reasonable in view of the similarity of the two curves. One can only say, however, that the fact that the damage and recovery pattern following a second dose was the same as that following the initial dose indicates that there was complete metabolic recovery only with respect to the process of spermatogenesis. More complete cytological and genetic studies would be necessary to throw light on this question.

The length of time (between 14 and 21 days) required for the spermatogonia to recover from the mitosis-inhibiting effect of irradiation is rather striking. If it is true that the division of spermatogonia to reproduce their kind is really similar to the division of somatic cells, a similar irradiation effect should be observed in other tissues. Experiments have been initiated to determine whether this is the case.

SUMMARY

LAF₁ mice were exposed to 50 r, 100 r, 200 r, 300 r and 400 r of 186 kv. roentgen rays directed to the whole body at 3 months of age. The course of damage and recovery of the testes was studied by observing the weights of the fresh testes and by quantitative histological analysis of spermatogenic elements. This damage-recovery pattern was also studied in terms of weights of fresh testes following a second 200 r roentgen irradiation of the whole body after the testes had recovered from an initial dose; and in terms of weights of fresh testes following exposure to a 10 mev source of roentgen rays for comparison with 186 kv. roentgen rays. From these studies the following tentative conclusions have been drawn.

1. The course of loss and recovery of weights of testes and the histological appearance during this damage and recovery period can be explained on the basis of a selective effect of radiation on a single stage of spermatogenesis, the spermatogonia.
2. This effect of the doses used, on a single stage of spermatogenesis, is not one of cell death but is one of inhibition of division of spermatogonia to form daughter spermatogonia.
3. Resting spermatogonia present at the time of irradiation appear to develop normally into spermatocytes, spermatids and spermatozoa.
4. The process by which the spermatogonia divide into daughter spermatogonia and the secondary spermatocytes into spermatids is generally considered to be morphologically the same as somatic mitosis in general. Such morphologic similarity was noted in the present experiments. The observation that there is a difference in the sensitivity of these two cell divisions to ionizing radiation suggests that they are physiologically different, although morphologically similar.

5. The course of damage and recovery of the testis following exposure to a second dose of radiation is the same as that of damage and recovery following an initial exposure.

6. The pattern of loss and recovery of weights of the testes of mice exposed to 10 mev roentgen rays is the same as that following exposure to 186 kv. roentgen rays.

Because of its sensitivity and the reproducibility of results, the testis is almost without parallel as a mammalian test organ for measuring the biologic effect of ionizing radiations. It will no doubt prove to be a valuable tool for subsequent studies on the relation between biologic effect and physical dose of ionizing radiations.

TESTICULAR INTERSTITIAL CELL TUMORS IN HYBRID MICE GIVEN TRI-P-ANISYL CHLOROETHYLENE

W. U. GARDNER, Ph.D.

AND

J. BODDAERT *

NEW HAVEN, CONN.

TESTICULAR interstitial cell tumors occur rarely in untreated mice of most strains,¹ but in mice of some strains they occur frequently subsequent to the injection of estrogens—especially in mice of the Strong A and the C strain.² Interstitial cell tumors appeared in seven mice of the JK strain and in one mouse of the C₃H strain that had been subjected to prolonged treatment with triphenylethylene (Gardner, 1943, table 1).

The strain-limited tendency for interstitial cell tumors to appear among estrogen-treated mice and the fact that they appeared among hybrids obtained by crossing a susceptible strain with a resistant strain lead to the assumption that susceptibility is genetically determined,^{1c} but the experiments, although extensive, were not conclusive, as studies were not carried out on mice beyond the first hybrid generation, because other types of tumors appearing at early ages prevented the desired survival of the experimental animals and because quantitative differences in the amount of estrogen administered did not range widely enough.

From the Department of Anatomy, Yale University School of Medicine.

* Belgian-American Educational Foundation Fellow on leave of absence from the department of pathology of the University of Ghent, Belgium.

This investigation was supported by grants from the Anna Fuller Fund, the Jane Coffin Childs Memorial Fund for Medical Research and the United States Public Health Service.

1. (a) Athias, M.: *Arg. Pat.* **17**:397, 1945. (b) Athias, M., and Furtado-Dias, M. T.: *Ibid.* **13**:381, 1941. (c) Gardner, W. U.: *Cancer Research* **3**:757, 1943.

2. (a) Bonser, G. M.: *J. Path. & Bact.* **54**:149, 1942; (b) **56**:15, 1944. (c) Bonser, G. M., and Robson, J. M.: *Ibid.* **51**:9, 1940. (d) Hooker, C. W.; Gardner, W. U., and Pfeiffer, C. A.: *J. A. M. A.* **115**:443, 1940. (e) Hooker, C. W., and Pfeiffer, C. A.: *Cancer Research* **2**:759, 1942. (f) Shimkin, M. B.; Grady, H. G., and Andervont, H. B.: *J. Nat. Cancer Inst.* **2**:65, 1941.

TABLE 1.—Summary of Investigations of Testicular Tumors of Mice

Author	Mouse Strain or Stock	Esterogens Used*	Mice	Method of Application	Dose	Duration	Tumors	Mice Showing Hypertrophy and Hyperplasia
Burrows, H.: J. Path. & Bact. 42: 161, 1936.	Stock	7 different	102	Peritoneous	Variable	5-9 mo.	..	77
Gardner, W. U.: Anal. Rec. 67: 49, 1930; 68: 139, 1937.	A CBA CBA F	5 different	15 8 8 8	inj. in oil inj. in oil inj. in oil inj. in oil	Variable Variable Variable Variable	2.5-8 mo. 4-9 mo. 4-9 mo. 4-9 mo.	13 1 0 0
Hooker, Gardner, Pfeiffer, ²⁴ 1939.	A	STE EB	...	inj. in oil inj. in oil	1.9 mo. 1.9 mo.	1 1	7 7
Bosmer and Robson, ²⁵ 1940.	RH RH CBA CBA	TPE EDP TPE EDP	30 31 30 30	inj. in oil inj. in oil inj. in oil inj. in oil	3 mg. wkly 3 mg. wkly 3 mg. wkly 3 mg. wkly	30-59 weeks 30-59 weeks 30-59 weeks 30-59 weeks
Shimkin, Grady and Andervont, ²⁷ 1941	C	STE STE	62 30	Pellets Pellets	Variable Variable	6-11 mo. 7 mo.	13 2
Hooker and Pfeiffer, ²⁸ 1942.	A A A A	EB EB STE TPE	9 40 47 31	inj. in oil inj. in oil inj. in oil inj. in oil	16.47 wkly 507 wkly 6.25 mg. wkly 3 mg. wkly	6-14 mo. 6-14 mo. 6-14 mo. 30-59 weeks	3 7 29 21 5
Bosmer, ²⁸ 1942.	JK	TPE	13	inj. in oil	2.5 or 5 mg. weekly	6-19 weeks	7	..
Gardner, W. U.: Cancer Research 3: 9, 1943.	A C ₃ H C ₃ H C ₃ H C ₃ H C ₃ H N	TPE TPE TPE TPE TPE TPE TPE	17 14 6 5 5 5 5	inj. in oil inj. in oil inj. in oil inj. in oil inj. in oil inj. in oil inj. in oil	2.5 or 5 mg. weekly 2.5 or 5 mg. weekly 2.5 or 5 mg. weekly 2.5 or 5 mg. weekly 2.5 or 5 mg. weekly 2.5 or 5 mg. weekly 2.5 or 5 mg. weekly	15-18 weeks 9-22 weeks 11-13 weeks 18-25 weeks 15-21 weeks 15-21 weeks 15-21 weeks	7 1
Bosmer, ²⁸ 1944.	W. L. IPB	TPE TPE	39 30	inj. in oil inj. in oil	3 mg. wkly 3 mg. wkly	30-59 weeks 30-59 weeks	3 4	21 14
Athlas and Furtado-Dias, ¹⁸ 1941.	H	None	7	7?	..
Athlas, ¹⁸ 1945.	H	None	109	42?	..

* STE indicates stilbestrol; EB, estradiol benzoate; TPE, triphenylethylene; EDP, estradiol dipropionate.

The synthetic estrogen tri-p-anisyl chloroethylene is more active biologically than is triphenylethylene. Biologically it behaves as a pro-estrogen, although the S/L ratio^{3a} is relatively low.^{3b} It is stored in large amounts in the body fat for appreciable periods. The appearance of interstitial cell tumors among hybrid mice was observed when tri-p-anisyl chloroethylene was administered, although such tumors had not been observed previously when mice of the same stock were treated with another estrogen.⁴

MATERIALS AND METHODS

The mice used in this investigation were hybrids designated CC₁ in this laboratory (C₅₇ × CBA ♂). Throughout the experiment they were maintained in an air-conditioned room (temperature 72-76 F. and humidity 50-60 per cent). They were fed a prepared food (purina® fox chow) and water ad libitum.

Ninety-two mice from 28 to 52 days of age at the start of the experiment were given weekly subcutaneous injections of 50 micrograms of tri-p-anisyl chloroethylene⁵ dissolved in 0.05 cc. of sesame oil (TACE 1®). After the experiment had been in progress from four to six months 49 of the mice were given weekly 100 micrograms of the estrogen in 0.05 cc. of sesame oil (TACE 2®).

All the mice were examined daily until tumors developed, until death or until their general condition declined. At necropsy all organs were examined for gross lesions, and tissues or organs showing lesions, in addition to the genital organs and adrenal glands, were preserved in Bouin's fluid for histological study. In addition the mammary glands of 14 representative mice were studied as whole mounts.

In previous experiments 17 males of the same genetic origin and 25 reciprocal hybrid mice were given estradiol benzoate,⁴ and none had testicular tumors, although 13 survived for more than 500 days.

OBSERVATIONS

Testes.—Of the 92 male mice, 76 survived 20 months or more and 46 had testicular tumors at death (table 2 and fig. 1). The 46 tumorous testes were classified in two groups, testes of normal size or reduced size, and testes showing enlargement in excess of normal. The testes of the first type were of normal size or were from one-half to two-thirds normal size. They were never larger than normal size. The tunica albuginea was usually considerably thickened.

3. (a) C. W. Emmens (J. Endocrinol. 2:444, 1941) defines the "S/L (systemic: local) ratio" as "the ratio of the median effective subcutaneous dose (that required to give 50% of positive responses) to the corresponding intravaginal dose." (b) Thompson, C. R., and Werner, H. W.: Federation Proc. 4:137, 1945; 5:208, 1945.

4. Gardner, W. U.: Cancer Research 1:345, 1941.

5. The tri-p-anisyl chloroethylene was supplied by the W. S. Merrel Company, Research Laboratories, Cincinnati, through the courtesy of Dr. Harold W. Werner, director, Scientific Laboratories.

The testes of the second type ranged in size from 0.8 cm. to 2.2 cm. in diameter. These testes were spherical or pear shaped, with smooth or nodular surfaces, and often showed prominent distended blood vessels. Through the tunica albuginea the tumorous tissue appeared as white masses in which characteristic yellowish spots or streaks were present. Areas of hemorrhage and necrosis appeared reddish brown.

Of the 46 mice having testicular tumors, 22 had unilateral tumors and 24 had bilateral tumors. Twenty-five animals had tumors that enlarged the testes. No correlation between the size of the tumor and the period of treatment was demonstrable; large tumors were evident after 600 days and small tumors were found after 850 days (table 2). Most of the large tumors appeared in mice older than 700 days (fig. 2).

TABLE 2.—*Testicular Interstitial Cell Tumors Among Ninety-two Hybrid Mice (C₅₇ × CBA δ) Given Weekly Subcutaneous Injections of Tri-*p*-Anisyl Chloroethylene*

Group	Average Age at Death, Days	Range of Age at Death	Mice	Number with Seminiferous Vesicle Stimulation
With tumors.....	781	605-854	46	..
With tumors enlarging testes *.....	787	605-850	25	21
With nodules not enlarging testes †	787	605-854	21	14
With bilateral tumors.....	771	605-849	24	..
Without tumors.....	627	344-846	46	..

* These include two tumors not examined histologically.

† These include one tumor not examined histologically.

Most of the mice had testes that contained a single small nodule or a single large mass. In only rare instances could multiple nodules or masses be demonstrated. The tumor cells comprising these nodules or tumors were of three distinct types, as already described by Hooker and Pfeiffer.²⁶

In the 21 mice with tumors recorded as small and not enlarging the testis (table 2) the nodules differed in size and occupied from one-fourth to almost the entire testis. The smallest nodules were centrally or eccentrically situated opposite the hilus in sections where the hilus could be seen. The nodules were fairly well circumscribed, although in seven testes tumor cells extended peripherally among the seminiferous tubules. A definitive connective tissue membrane was never encountered.

The tumor cells in 12 of these nodules were of a single type. They were densely packed, round or polygonal in shape, and were arranged in broad sheets or irregular cords. The cells forming these tumors were designated type 1. They were two or three times the size of the normal Leydig cell and had a characteristic, highly vacuolated cytoplasm,

with slightly eosinophilic granules. The vesicular nucleus was round or oval, centrally located, and contained one or two nucleoli. Large amphophilic intranuclear inclusions were frequently seen. Mitotic figures were rare, but a moderate number of cells had two nuclei, suggesting amitotic division. The cytoplasm of some of the cells was characterized by a highly vacuolated peripheral exoplasm and a granular acidophilic endoplasm containing the nucleus. Scattered among these cells were occasional moderately or completely pigmented cells with one or more shrunken nuclei. The capillary networks penetrating the tumor masses

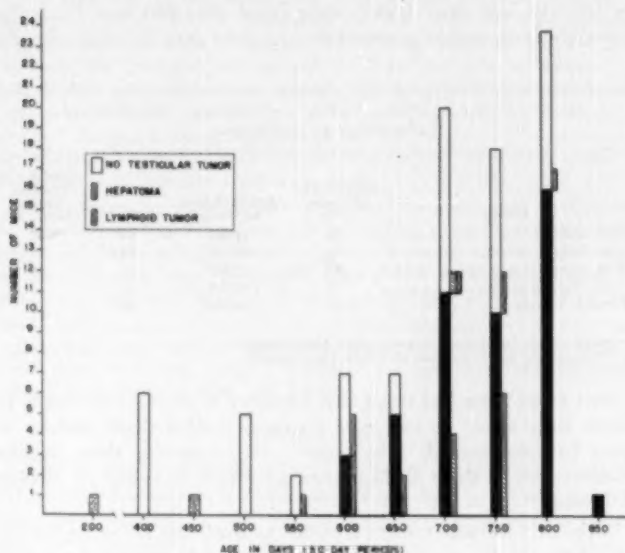


Fig. 1.—Incidence of testicular, hepatic and lymphoid tumors in hybrid mice given tri-p-anisyl chloroethylene.

were either distended or collapsed and in immediate contact with the cells. The nodules contained remnants of spermatid tubules that had their walls either reduced to a basement membrane containing an eosinophilic staining fluid or lined by Sertoli cells and spermatogonia. In the nontumorous portions of the testes the peripheral tubules often revealed active spermiogenesis.

Between the two extreme conditions—tubules reduced to a basement membrane and tubules with complete spermiogenesis—all degrees of testicular atrophy occurred, even in the same testis. Agglutination of

spermatozoa, coalescence of spermatids into multinuclear cells, and cellular debris intermixed with homogeneous eosinophilic droplets were observed frequently. Atrophic changes were usually more obvious in the tubules adjacent to the tumorous areas. However, the tubules in this location occasionally showed spermiogenesis, although the tubules in the periphery contained no spermatozoa.

Two types of cells were seen between the seminiferous tubules: a highly vacuolated cell not unlike tumor cell type 1, and a mononuclear or multinuclear brown-pigmented cell, the latter probably arising by fusion of two or more mononuclear pigmented cells. All stages of transition between these two cell types were present.

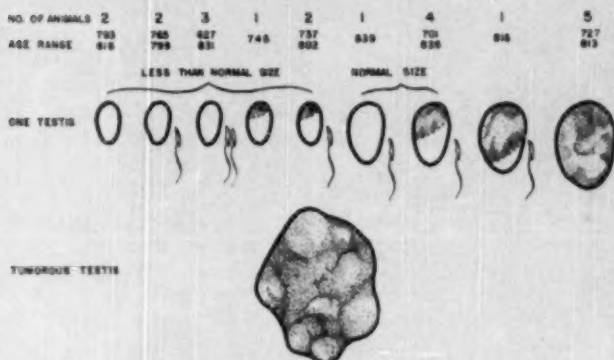


Fig 2.—Condition of the contralateral testis in mice with at least one large testicular tumor.

In the five tumors recorded as enlarging the testis and composed of cell type 1 (table 3) the microscopic appearance was essentially similar to that seen in the small nodules of the 12 tumors already described. The testicular parenchyma was practically completely replaced by tumor cells. In only a few instances were tubules in which spermiogenesis was evident seen at the periphery.

The arrangement in which cells of the tumors formed cords or alveoli, surrounded by a thin connective tissue membrane, was more apparent at the periphery than elsewhere. Some of the alveoli were lined only by cells with a foamy appearance (type 1), and others by brown-pigmented cells with shrunken nuclei, and still others showed lining composed of cells of both types (fig. 3 *A* and *D*).

More frequently among the larger tumors, degenerative changes were noted in some of the cords or the alveoli; intercellular spaces

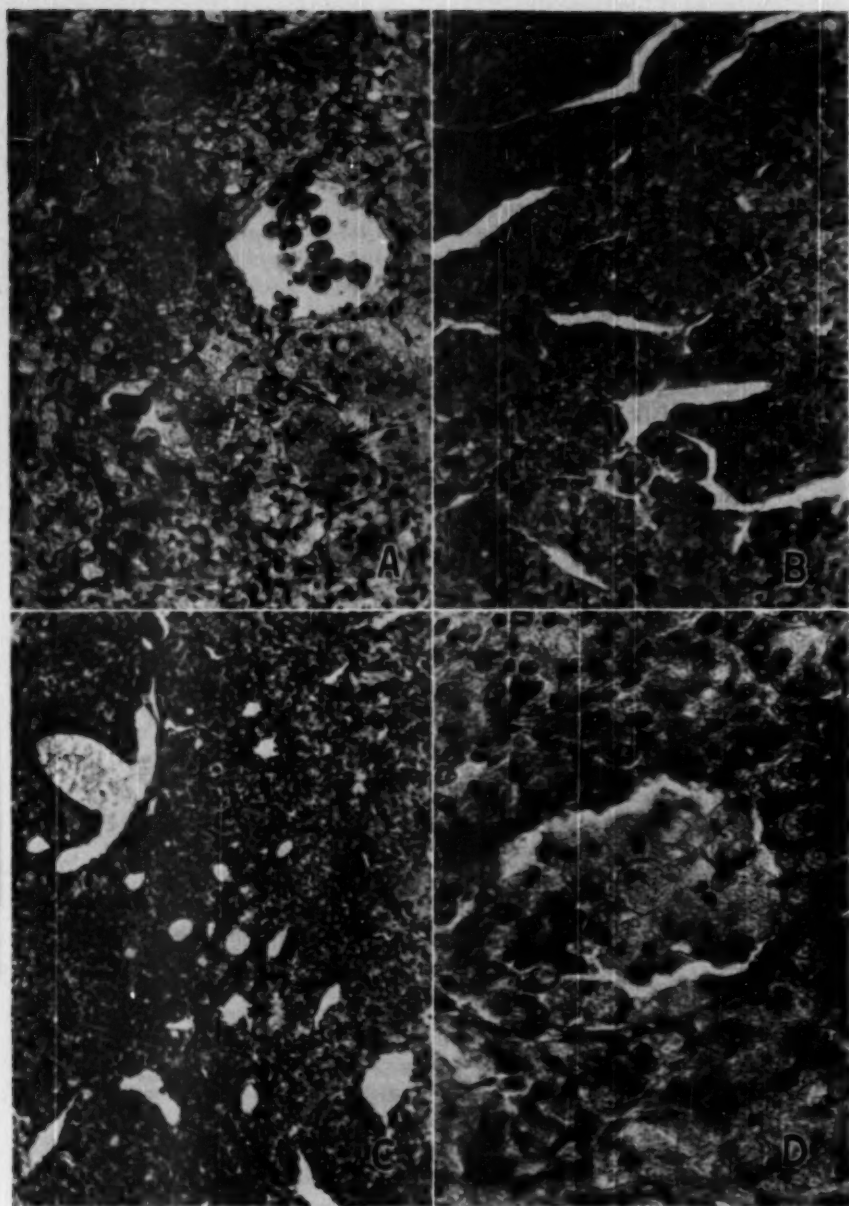


Figure 3
(See legend on next page)

appeared, forming lumens in which degenerating cells intermingled with an eosinophilic staining fluid (fig. 3*A*). These cavities were often large—approximately 0.5 mm.—and were sometimes filled with blood. Small areas of necrosis were frequent, and one testis contained a large area of coagulation necrosis.

Eight small tumors and 14 large tumors were composed of cells of two types (table 3). In addition to the cells of type 1, described above, these tumors contained cells slightly larger than the normal Leydig cell. In these cells vacuolation was absent or minimal and the acidophilic cytoplasm was homogeneous or coarsely or finely granular (fig. 3*B*). The variations in nuclear size, amount of chromatin and number of intranuclear inclusions were more pronounced in these cells than in cells of type 1. Mitotic figures occurred more frequently. The cells of the two types were either mixed together or grouped in discrete nodules in the tumor. The number of cells of each type comprising the different tumors was variable. Transitional cells intermediate between types 2 and 1 were seen. Sections of tumors containing mostly cells of type 2 always stained deeply and contrasted strongly with the sections showing only cells of type 1.

Cells of a third type were present in four of the large tumors (table 3 and fig. 3*C*). They were similar to those described by Hooker and Pfeiffer²⁰ as being one-half the size of the normal Leydig cell and having more or less basophilic cytoplasm. In seven large tumors the histological structure differed from that described above. In these the cells of type 2 and type 3 were arranged in trabeculae or anastomosing cords of varying caliber. Each cord was delimited by a thin connective tissue lamella or by endothelium-lined blood channels (fig. 3*B*). This structural arrangement comprised either a portion or the whole of the tumor. In two instances the small cells (type 3) were found in strand-like arrangement interspersed between cells of type 1.

EXPLANATION OF FIG. 3

Fig. 3.—*A*, photomicrograph of the testicular tumor of a mouse given 50 micrograms of tri-*p*-anisyl chloroethylene (TACE*) weekly for 99 weeks. It is composed predominantly of cells of type 1, some of which contain a brown pigment. The small cyst is lined by tumor cells and contains a few isolated cells. Approx. $\times 200$.

B, photomicrograph of the testicular tumor of a mouse given 50 micrograms of TACE* weekly for 21 weeks and 100 micrograms weekly for the following 56 weeks. The area shown is composed predominantly of cells of type 2, arranged in coarse trabeculae. Approx. $\times 200$.

C, photomicrograph of the testicular tumor of a mouse given 50 micrograms of TACE* weekly for 12 weeks and 100 micrograms weekly for 97 weeks. The area shown contains predominantly cells of type 2 with areas of type 3. Approx. $\times 100$.

D, same tumor as shown in *C*, revealing transition of small cells to larger cells (type 1). Approx. $\times 400$.

Eleven of the large tumors revealed enormously distended blood channels or cystic cavities containing red blood cells. Hemorrhage and hemosiderin-containing phagocytes were associated frequently with the cysts. Areas of necrosis were also present. In five tumors areas of erythropoiesis and myelopoiesis were seen. Fifteen of the tumors showed neoplastic invasion of the tunica albuginea. Metastasis was observed in the left perirenal lymph node in two animals.

TABLE 3.—*The Histological Types of Interstitial Cell Tumors Among Hybrid Mice (CC₁) Given Tri-p-Anisyl Chloroethylene and Evidence of Androgen Production as Determined by Size of Seminal Vesicles*

	Type 1	Types 1 and 2	Types 1, 2 and 3	Types 2 and 3
Large tumors.....	5	14	8	2
Androgen.....	4	10	2	1
No androgen.....	1	4	..	1
Small tumors.....	12	8
Androgen.....	7	6
No androgen.....	5	2

TABLE 4.—*Effect of Large and Small Doses of Tri-p-Anisyl Chloroethylene on the Development of Testicular Interstitial Cell Tumors in Hybrid Mice (CC₁)*

Weekly Dose, Micrograms	Size of Tumors	Mice	Average Age at Death, Days	Range of Age at Death
50	Enlarging testis.....	13	759	627-839
	Not enlarging testis.....	9	790	697-854
	Bilateral tumors.....	9	793	797-849
50 and 100	Enlarging testis.....	10	754	605-824
	Not enlarging testis.....	12	754	633-817
	Bilateral tumors.....	15	759	633-813

In all animals having small testicular tumors composed of cell type 1 the presence or the absence of spermiogenesis was not related to the amount of tri-p-anisyl chloroethylene injected, to the size of the testis, to the bilaterality of the tumor or to the size of the seminal vesicles (table 4).

In all animals which had small tumors composed of cells of types 1 and 2 and in which the size of the testis was normal, spermiogenesis was present only in that group receiving 50 micrograms of tri-p-anisyl chloroethylene weekly. In this group the age range was from 781 to 826 days. No spermiogenesis was seen in the animals receiving 100 micrograms of the estrogen weekly and in which the age ranged from 752 to 814 days. The seminal vesicles of these mice were of either castrate or normal size. Among the animals having large testicular

tumors and ranging in age from 600 to 839 days only one did not show spermiogenesis in one or both testes, and all had seminal vesicles of normal size or enlarged.

In the study of the 46 mice with nontumorous testes 18 testes were examined histologically; the ages of the animals ranged from 450 to 800 days. These testes were from two-thirds to one-fourth the normal size. Spermiogenesis occurred only in the testes of the mice that were 755 to 799 days old and that had received 50 micrograms of the estrogen weekly.

The reduction in size of the testes was due mainly to a reduction in size of the seminiferous tubules. The intertubular spaces were occupied

TABLE 5.—*Tumors Other Than Testicular Appearing Among Ninety-two Hybrid Mice (C₃H ♀ × CBA ♂) Given Weekly Injections of Tri-p-Anisyl Chloroethylene*

Type of Tumor	Mice with Tumor	Average Age, Days	Age Range	Mice with Testicular Tumors
Hepatoma.....	10	730	627-813	10
Lung adenoma.....	5	731	627-854	3
Lymphoma.....	13	...	344-781	..
Reticulum cell sarcoma.....	5	604	600-783	3
Lymphosarcoma.....	5	713	600-771	2
Histiocytoma.....	1	562	0
Leukemia.....	2	870	344-696	0
Pituitary adenoma.....	5	730	536-826	2
Fibrosarcoma.....	2	790	739-841	0

by brown-pigmented interstitial cells, large, highly vacuolated cells, fibroblasts and occasional plasmocytes.

In four of the animals the complete lack of spermiogenesis and the proliferation of brown-pigmented cells were limited to half the testis, the other half showing active spermiogenesis, occasional brown-pigmented cells and a large number of highly vacuolated cells, which were probably hypertrophied Leydig cells. In these animals the seminal vesicles were of the castrate type.

Lymphoid Tissues.—Of the 92 male hybrid mice (C₃H ♀ × CBA ♂) that survived eight months or more after weekly injections of tri-p-anisyl chloroethylene, 13 had lymphoid tumors, and five of these tumors occurred in animals also having testicular tumors (fig. 1 and table 5).

Mesenteric and mediastinal lymph nodes, spleen, liver and kidneys were involved to different degrees in the different mice. The tumors were classified microscopically as reticulosarcoma, lymphosarcoma, leukemia and histiocytoma. Reticulosarcomatous cells were found in the testes in two instances, once in a tumorous testis and once in a non-tumorous testis.

Liver.—Hepatoma occurred in 10 animals that had testicular tumors, and in two animals without testicular tumors (fig. 1 and table 5). In these 12 mice hepatoma grossly ranged in size from small nodules to 2 cm. in diameter. It was seen either as a pedunculated, sometimes partially necrotic tumor or as a paler mass of tissue bulging from the liver and largely surrounded by normal hepatic tissue. It revealed a trabecular structure, the cells being arranged in anastomosing cords. The tumor cells were usually two to three times the size of normal liver cells. Binuclear cells and occasionally mitotic figures were seen.

Mammary Glands.—Mammary tumors were observed in none of the mice. Observations at the time of autopsy revealed mammary growth in all mice, and the glands of the 14 mice studied in detail after being prepared as gross mounts resembled those of mice given estradiol benzoate.³ The glands of two of the mice showed extensive alveolar development. In one mouse only 163 days of age and not included among the 92 reported, only ducts were present, but the over-all glands were large. The glands of the other 11 mice showed intermediate development of small lateral branches or alveoli. The presence of large testicular tumors did not modify the response of the mammary glands.

Pituitary Gland.—Although the pituitary glands of many of the mice were moderately enlarged (4-6 mg.), the glands of only five weighed in excess of 12 mg. and ranged in weight from 13.0 to 100.6 mg.

COMMENT

The failure of testicular interstitial cell tumors to appear in mice of similar genetic origin when given 16.6 or 50 micrograms of estradiol benzoate weekly⁴ and their appearance when tri-p-anisyl chloroethylene (TACE[®]) was given merit comment. The testes of mice given estradiol benzoate were all reduced in size, contained multinuclear masses of pigmented cells and in a few instances showed small areas of hypertrophied glandular interstitial cells. No tumors were observed, but only two male mice survived for more than 600 days. Three of seven mice given TACE[®] had testicular tumors at ages between 600 and 649 days; all the other interstitial cell tumors occurred in older mice. The possibility exists that the mice given estradiol benzoate did not survive sufficiently long to permit the development of testicular tumors. If such is the case, the potentiality for testicular tumors could be evoked by estrogens other than TACE[®] or related chemicals if they were administered adequately and under conditions that would permit prolonged survival. Experiments designed to determine whether or not the relative duration of survival when mice are exposed to different

estrogens could account for the different neoplastic responses of the testes would be extremely time consuming, even if they might indicate a difference in the tumorigenic response of an end organ.

Quantitative differences in effective levels of estrogens might be of as great or even greater significance than the duration of the experiment. The fact that the mice given TACE[®] survived so much longer than the mice given estradiol benzoate in the two amounts mentioned above indicates that they were subjected to relatively less estrogen in terms of pharmacologic effects. Extreme retention of urine and hydro-nephrosis were rarely encountered. Furthermore, whereas 83 per cent of the estradiol benzoate-treated mice had pituitaries weighing in excess of 12 mg. (chromophobe adenoma) only 5 per cent of the mice given TACE[®] had such tumors and the largest tumors were much smaller than those recorded in the former experiment. These observations, together with the less extensive resorption of the rami of the symphysis pubis, the less extensive hyperostosis of the long bones and the larger size of the nontumorous testes of some of the TACE[®]-treated mice, indicate that they were subjected to relatively lower levels of estrogen.

The assumption has been made that estrogens act indirectly on the testes in the production of interstitial cell hypertrophy, hyperplasia and tumors; that they might elicit a prolonged and heightened production of interstitial cell-stimulating hormone from the hypophysis, the latter being responsible for the formation of tumors.⁶ At the present time proof that TACE[®] produces effects on the hypophysis qualitatively different from those of other estrogens is lacking. The difficulties of obtaining with known methods of standardization quantitatively comparable levels of different estrogens are great. When TACE[®] is injected subcutaneously once weekly, 1 vaginal cornifying unit might produce effects quite different from those of the amount of estradiol or estradiol benzoate required to produce a vaginal response. Evidence is not available that TACE[®] is more effective in stimulating the production or the release of the interstitial cell-stimulating hormone of the pituitary.

The fact that interstitial cell tumors appear in testes transplanted intrasplenically in castrated rats⁷ again indicates that the pituitary hormones are responsible for their formation. Testicular tumors did not occur in intrasplenic testicular transplants in castrated mice.⁸

6. (a) Gardner, W. U.: *Anat. Rec.* **68**:339, 1937; (b) *Cancer Research* **8**:397, 1948. (c) Hooker and Pfeiffer.²⁰

7. Biskind, M. S., and Biskind, G. R.: *Proc. Soc. Exper. Biol. & Med.* **50**:1945. Twombly, G. H.; Meisel, D., and Stout, A. P.: *Cancer Research* **9**:596, 1949.

8. Li, M. H.; Pfeiffer, C. A., and Gardner, W. U.: *Proc. Soc. Exper. Biol. & Med.* **64**:319, 1947.

TACE* is stored in the body fat of rats. Ten milligrams per 100 Gm. was found in the body fat one day after rats had received three 5 mg. doses of TACE*; 2 mg. per 100 Gm. was found 10 days later.³ The possibility that TACE* is stored in the lipid-containing interstitial cells of the testis exists. Body fat storage also probably assures prolonged effectiveness of periodically administered doses.

The fact that small interstitial cell tumors or nodules appear in small or atrophic testes as well as in testes of approximately normal size and containing many tubules showing active spermiogenesis indicates that the spermiogenic function of the testis is unrelated to the formation of the tumors. In the nontumorous or partially tumorous testes of mice with one large testicular tumor the spermiogenesis may be due, in part, to androgen produced by the tumor.

Three testicular interstitial cell tumors have been observed in untreated mice in the colony of one of us (W. U. G.).^{1c} None have occurred in mice of the hybrid group employed in the present experiment.⁴ No testicular tumors have been observed in mice of the A strain, in which such tumors regularly occur after estrogen treatment. Athias^{1a} has, however, described tumors of the interstitial cells in untreated mice of one strain and commented that mice of other strains might show such lesions. Such has, however, not been true in this laboratory.

The working hypothesis based on the original data^{3b} that genetic influences transmitted by both males and females determined the predisposition toward testicular tumors subsequent to estrogen treatment, no matter what the mechanism—that is, whether acting directly on the testis or indirectly through the pituitary—is appreciably weakened by the observations presented. The probability of an "all or none" difference between strains is diminished. The difficulties of adequately demonstrating quantitative differences if they are genetically determined are great in problems of this type, especially when these differences may involve (1) the specific and organ (testis), (2) the intermediate effector glands (pituitary, thyroid or adrenal), (3) the degradation, excretion or metabolism of the hormone (liver, intestine or kidneys) or (4) the influence of environmental factors on any of the above (food, room temperature, cryptorchidism, etc.). On the other hand, if strain differences in response to TACE* exist, the final solution of the problem may be assisted.

Some additional morphological details have been reaffirmed or established by this investigation. The present observations reaffirm that the testicular interstitial cell tumor takes origin in testis in which the Leydig cells are largely depleted, and independently of the condition of the seminiferous epithelium. In agreement with Athias,^{1a, b} it is considered that the pigmented mononuclear or multinuclear cells are derived

from large glandular interstitial cells—the multinuclear, by a coalescence of the mononuclear. The present observation does not indicate that they are macrophages as such. Macrophages were numerous in some of the tumors, especially where necrosis or interstitial hemorrhage occurred, and could always be histologically distinguished from the pigmented cells of interstitial cell origin. The pigmented cells may phagocytose as shown by Hooker and Pfeiffer,²⁰ but the nice evidence that they are formed from the interstitial cells is quite convincing.

Although the tumors arose in mice of a different genetic constitution and in the presence of a different estrogen, they showed the same general range of histological structure as the tumors in mice of the A strain treated with esters of estradiol or diethylstilbestrol. The fact that but two of the tumors had extended to the perirenal lymph nodes may be significant. A larger number of tumors of similar size in mice of the A strain would have involved these nodes.²⁰ The occurrence of the tumors only in aged mice of the CC₁ groups, in contrast to their earlier appearance in mice of the A strain, may be of significance. The tumors in mice of the A and CC₁ groups were similar in the invasion of the tunica albuginea.

The fact that 10 of the 12 tumors of the liver (hepatoma) occurred among the 46 mice with testicular tumors may be of significance. The remaining two occurred among the 46 mice without testicular tumors. The difference in incidence cannot be explained on the basis of age alone.

The incidence of lymphoma was not significantly different from that in mice given estradiol benzoate,⁴ but the relative numbers of mice showing reticulosarcoma and histiocytoma were greater. The latter tumors appear in general in older mice, and the difference in incidence could be accounted for on the basis of age alone.

SUMMARY AND CONCLUSIONS

Forty-six of 92 hybrid mice (C₃H ♀ × CBA ♂) had testicular interstitial cell tumors after all 92 mice had been given weekly doses of 50 or 100 micrograms of tri-p-anisyl chloroethylene (TACE*) beginning at 28 to 52 days of age and continuing up to 850 days.

Twenty-five of the tumors enlarged the testes—up to 2.2 cm. The large tumors were associated with (1) a second large tumor of the contralateral testis, (2) a small tumor of the contralateral testis or (3) a nontumorous contralateral testis. Small tumors, not enlarging the testes, occurred in 21 mice.

Ten hepatic tumors (hepatoma) occurred in the 46 mice with testicular tumors and two in the 46 animals without interstitial cell tumors. Difference in survival does not account for the difference in incidence of hepatoma in the mice with and without testicular tumors.

Morphologically, the interstitial cell tumors were comparable to those induced in mice of the A strain. They consisted of cells of three main types. Fewer metastatic growths were observed (only two) than in mice of the A strain.²⁰

Either TACE[®] has a more marked tumorigenic effect on the testes, directly or indirectly, than does estradiol benzoate or the prolonged survival of the TACE[®]-treated mice permitted a realization of tumor potentialities not revealed previously. The hypothesis that there is a distinct genetic limitation of the potentiality for testicular tumors in estrogen-treated mice is weakened.

VASCULAR DEGENERATION IN HYPOTHYROIDISM

WILLIAM B. KOUNTZ, M.D.

ST. LOUIS

LITTLE attention has been paid to cardiovascular degeneration in conditions of hypothyroidism. In experiments on sheep complete thyroidectomy, followed by loss of hair and degenerative changes of the skin, has been associated with capillary degeneration. Students of anatomy, however, have paid little attention to the correlation of hypothyroidism and degenerative vascular disease, particularly that of the larger blood vessels.

The findings of chromatic degeneration and rupture of the aorta following thyroidectomy in a series of three cases led Kountz and Hemplemann¹ to a study of the relationship of arterial degeneration in hypothyroidism. On anatomic study, idiopathic aortic and arterial degeneration were found. Although the three cases were strikingly similar, the possibility of coincidence could not be excluded. Since our observations,¹ Foster and Barr² have reported one case with definite myxedema and have established a similar phenomenon. In addition, it has been possible to study one other case of thyroidectomy and emphysema and 13 cases of clinical hypothyroidism which was determined before death, and comparable changes have been found. Anatomic observations were made after postmortem examination in all cases.

The fourth case of total thyroidectomy is that of H. H., aged 54 at death. The discharge note from Barnes Hospital, May 10, 1934, sums up the patient's state:

He presented the picture of rather advanced emphysema, for which total thyroidectomy was performed. After the operation the patient felt better and was more comfortable at rest. The basal metabolic rate on admission was +12, on discharge -17, per cent, and the blood cholesterol was 180 mg. per 100 cc. One year later the basal metabolic rate was -24 per cent and the blood cholesterol 260 mg. The cholesterol in 1938 was 210 mg. and the basal metabolic rate was -30 per cent.

From the Division of Gerontology, Washington University School of Medicine, and the St. Louis City Infirmary and Infirmary Hospital, with autopsies from the Snodgrass Laboratory of the City of St. Louis.

1. Kountz, W. B., and Hemplemann, L. H.: *Am. Heart J.* 20:599-610, 1940.

2. Foster, M., and Barr, D.: *J. Clin. Endocrinol.* 4:417-426, 1944.

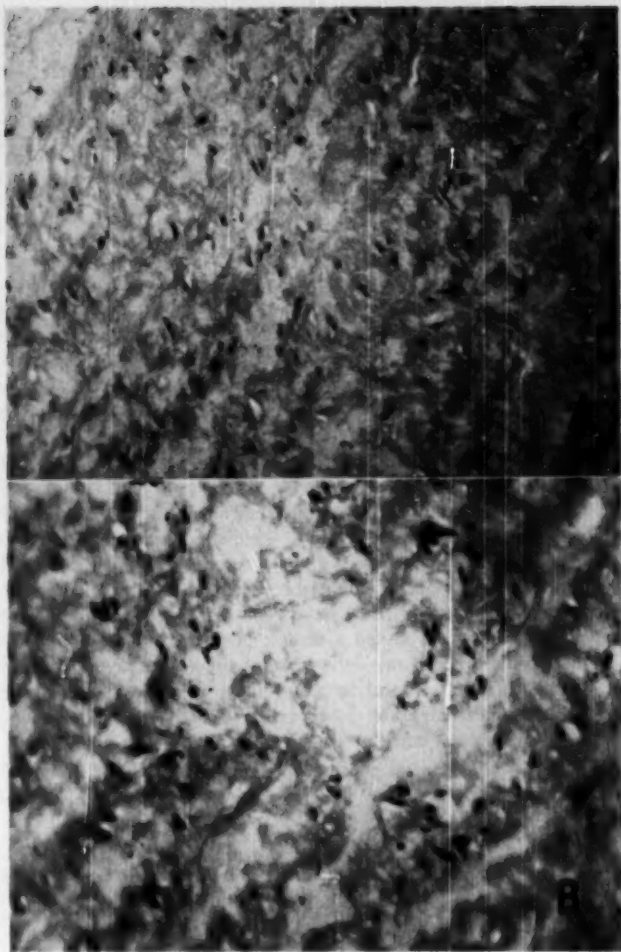


Fig. 1.—*A*, loss of muscle outline and accumulation of the myxoid material between the muscle bundles ($\times 220$). *B*, the same process of idiopathic cystic degeneration at a higher magnification ($\times 370$). One notes the granular nature of the material. The patient's thyroid gland had been removed because of cardiac decompensation and hypertension. He died of a ruptured aorta approximately six months after the operation.

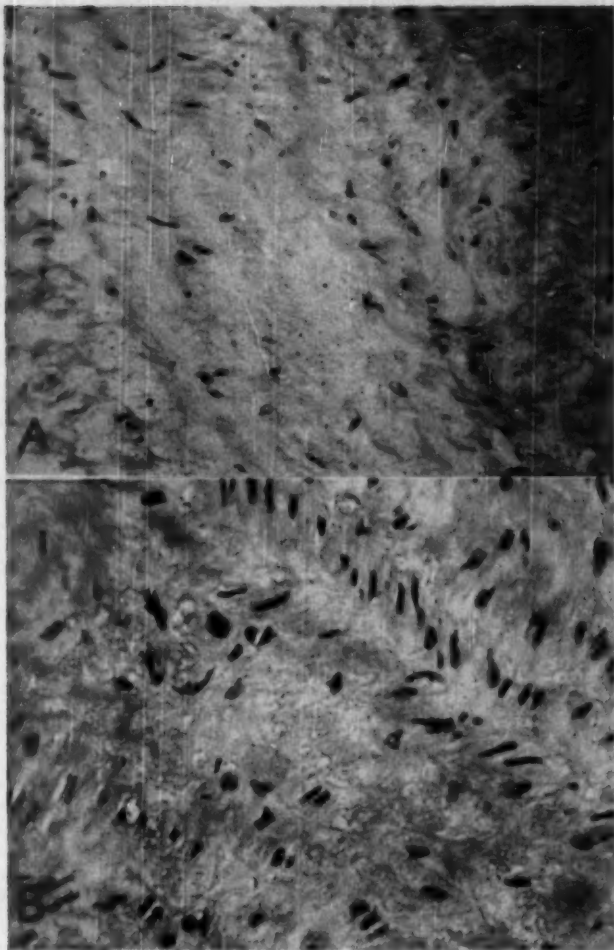


Fig. 2.—*A*, rather advanced infiltration and destruction of the media in idiopathic cystic degeneration ($\times 220$). The thyroid gland had been removed because of hypertension and cardiac failure, and the patient lived approximately three years postoperatively. Thyroid U. S. P. was taken about three fourths of that time. *B*, higher power magnification ($\times 370$) of the infiltration and destruction of the media represented in *A*.

Washington University Autopsy no. 8293 presented the following findings:

Heart: Gross Findings.—The heart is somewhat large, but not extremely so, for a body of this size. It weighs 350 Gm. The epicardium appears somewhat opaque, but it is smooth and moist on the surface. There is a large amount of subepicardial fat which, however, is not very thick in any one spot. The endocardium is relatively normal except for the outer wall of the right ventricle, where there is some opacity of the subendocardial tissue and two small white fibrous patches on the interventricular wall in the left ventricle. The mitral valve appears somewhat thickened and white. It is not as resilient as normal, but there probably is not enough change to have interfered with valve function during life. The coronary arteries are sclerotic. The right coronary artery is occluded 1 cm. from its orifice, this process being precipitated perhaps by the marked roughening and calcification of the fatty plaques of the intima and a small tear in the vessel wall. At the

TABLE 1.—Observations on Four Persons Whose Thyroid Glands Had Been Removed for Therapeutic Reasons and Who Died with a Cardiovascular Condition

Name	Sex	Age	Autopsy	Evidence of Thyroid Deficiency	Av. Basal Metabolic Rate	Clinical Signs of Hypothy- roidism	Anatomic Evidence of Arteriosclerosis		State of Thyroid at Autopsy
							Intimal	Medial	
K. K.*	F	39	5061	Thyroidectomy, complete	+39 before operation; —7 after	None noted	Minimal	Advanced	None found
H. C.*	M	25	6897	Thyroidectomy, complete	+33 before operation; —4 after	None	Minimal	Advanced	None found
W. B.*	..	39	6060	Thyroidectomy, complete	+33 before operation; —18 after	Slight increase of weight	None	Advanced	None found
H. H.†	M	54	8293	Thyroidectomy, complete	+12 before operation; —24 one year after	Moderate	Moderate	Advanced	None found

* The first three patients were relatively young and had little atheromatosis at postmortem examination.¹ Each of the three died of a ruptured aorta. Each had severe hypertension and impending cardiac failure.

† The case of H. H. has not been previously reported. He had severe emphysema. He led a sheltered life and died of coronary thrombosis. He had advanced intimal and medial change. He died about five years after his thyroid gland had been removed.

extreme left margin of the left ventricular wall the muscle is extremely scarred with an old process. The myocardium in this area measures 0.7 mm. and is very irregular as compared with the normal thickness of 17 mm. elsewhere. The myocardium of the right ventricle appears whitened and opaque. The measurements of the tricuspid, pulmonary, mitral and aortic valves are 135, 78, 84 and 80 mm., respectively. The right ventricular wall measures 2.5 mm. in thickness, and the left ventricular wall is 14 mm. in thickness.

Lungs.—The lungs appear like a limp rag on each side of the mediastinum. On the surface there are innumerable emphysematous blebs. Two blebs on the diaphragmatic surface of the lower lobe of the left lung appear cystic and measure from 10 to 15 cm. in diameter. No area for escape of air is noted. There is marked anthracosis of both lungs. The lungs appear firm and inelastic, and very irregular. There is some crepitant tissue in the anterior portions of the lungs. The upper lobe of the right lung is fairly firmly adherent to the parietal reflection of the pleura. On the left there are enormous calcified tracheobronchial lymph

nodes measuring 2.5 cm. in length, 2 cm. in width and 1.5 cm. in thickness. The bronchial lymph glands leading into the lower lobe on the left contain small calcified tubercles.

Vessels.—The pulmonary artery appears average in caliber and structure. The aorta, however, is remarkable in the degree of arteriosclerosis present. Beginning at the arch of the aorta and proceeding down the abdominal aorta, there is an increasing amount of plaquing of the intima. Little of the intimal coat is uninvolved. Many of the plaques are ulcerated. One such plaque just beneath the level of the branch of the inferior mesenteric artery measures 3 cm. in diameter and is

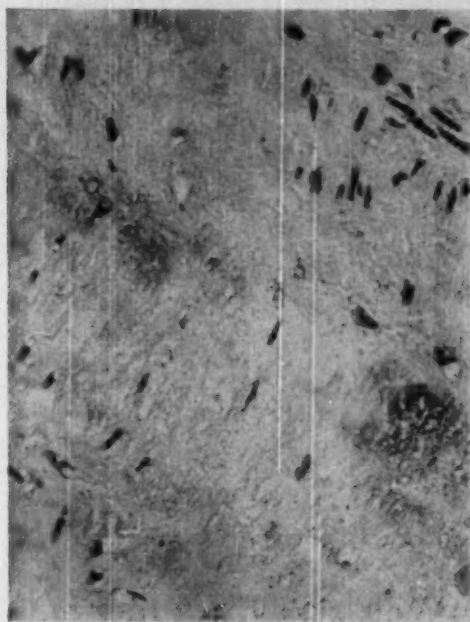


Fig. 3.—Advanced degenerative change occurring in the media with some calcium deposits ($\times 370$). The patient's thyroid gland had been removed because of emphysema, and he lived for five years postoperatively. He lived a quiet, inactive life and was unable to tolerate thyroid. He died of coronary thrombosis.

completely ulcerated. The surface is covered with a yellow friable material. In this particular plaque there is little calcium. Other plaques, however, are entirely composed of calcium, or where there is ulceration in the center, the area of ulcerated calcified intima is replaced by a blood clot at various stages of organization.

Heart: Microscopic Observations.—A section of the left ventricle shows a diffuse and moderately extensive focal fibrosis, with areas of degenerating muscle cells present in the myocardium. There is an extensive thickening of the media

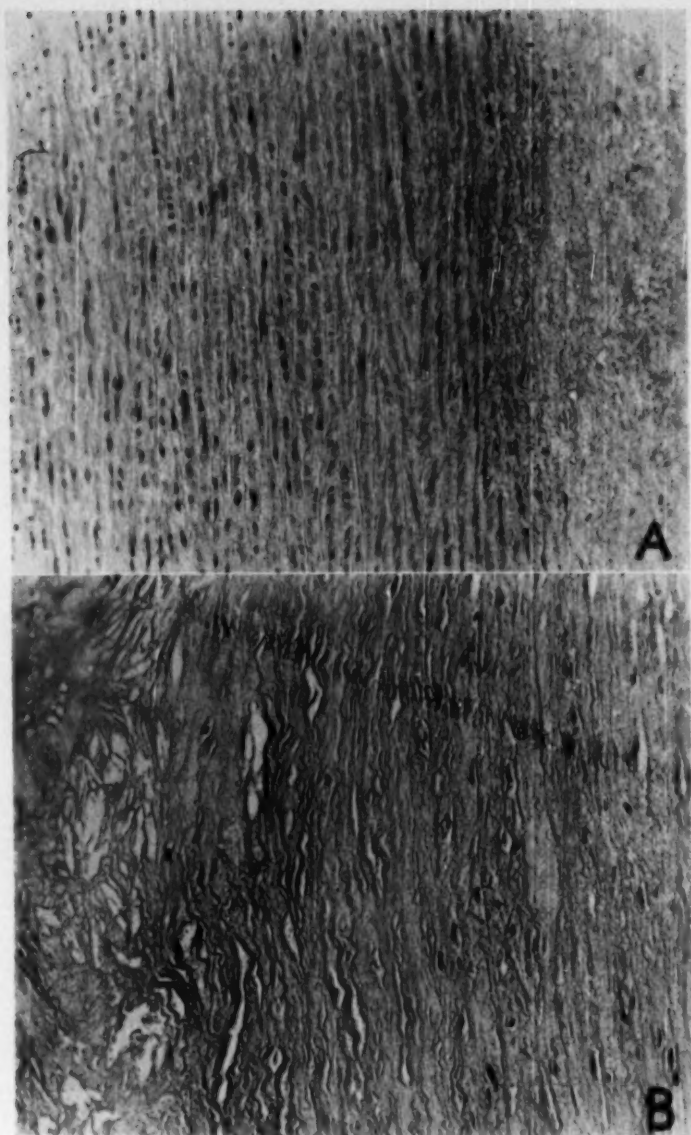


Fig. 4.—*A*, aortic wall ($\times 200$) of a 76 year old person whose average basal metabolic rate for the five year period before death was normal. Note particularly the degenerative change toward the intimal surface and the normal appearance of the media. The degenerative changes along the intimal surface were classified as moderate. *B*, aorta ($\times 200$) of a person, aged 60, whose metabolism for at least five years before death was persistently low, from -20 to -35 per cent. One notes particularly the marked deposit of cholesterol toward the intimal surface (at left), which extends well into the media. Notice also the swelling and separation of the muscle fibers and the loss of the outline of these fibers in contrast to *A*.

of a medium-sized coronary artery. An enormous thickening of the intima fills almost three fourths of the lumen of the right coronary artery, bulging in from one side, and this tissue is continuous with and has the same structure as the thickened intima of the remainder of the wall. The pale, acellular, collagenous appearance is identical. Irregular spaces are found between the margin of this tissue and the media of the artery.

Aorta: Microscopic Observations.—A thickened intimal layer takes up almost a third of the thickness of the wall in places, but varies considerably. Subjacent to the rather uniform layer of the intima is an irregular, acellular, acidophilic layer, showing irregular vacant spaces, and areas of calcification promiscuously intermingled with areas showing the shape of cholesterol crystals. The elastic tissue is frayed and broken. The outer portion of the media, forming the other third of the wall, appears more normal, though it is abnormally acidophilic, is

TABLE 2.—Observations on Thirteen Persons Whose Oxygen Consumption Was Low*

Name	Autopsy	Age	Clinical Evidence of Arteriosclerosis	Av. Basal Metabolic Rate			Clinical Signs of Hypothyroidism	Degree of Arteriosclerosis	
				Low	Normal	High		Intimal	Medial
M. S.	8798	75	++	—8	None	Advanced	Moderate
C. S.	8609	69	++	+1	None	Moderate	Moderate
S. H.	8814	66	++	—94	++	Advanced	Advanced
P. Q.	8616	60	++	—8	None	Moderate	Moderate
I. C.	8639	69	—	—5	None	Moderate	Moderate
M. F.	8616	67	++	—27	+++	Advanced	Advanced
M. B.	8654	65	++	—30	+	Moderate	Advanced
E. H.	8772	69	+	—32	++	Advanced	Advanced
N. S.	8796	60	+	—23	+	Advanced	Advanced
E. J.	8805	70	+	—15	+	Advanced	Advanced
A. B.	8774	61	++	+2	—	Advanced	Moderate
A. H.	8783	76	+	+14	—	Moderate	Minimal
A. U.	8775	68	++	—18	—	Advanced	Advanced

* These persons were observed at the City Infirmary for years before death. Their basal metabolic rates and blood cholesterol levels were determined every six months. The figures reported represent the averages of the basal metabolic rates taken on the subjects.

invaded in places by the process described above and contains spaces running longitudinally between cell layers.

A group of City Infirmary Hospital patients (table 2) was studied for evidence of arterial degeneration and decreased rate of oxygen consumption.

COMMENT

Microscopic examination of various levels of the aorta was made post mortem in all instances. The essential change in both the athyroid patients and those with low metabolism was the interstitial accumulation of an amorphous basophilic material between the muscle fibers and elastic fibers of the media. The amount and the staining properties of this material varied with each section. The largest accumulations were usually found in the ascending portion of the aorta and in some instances in the abdominal portion. The fenestrated elastic membranes and muscle cells were in some instances so separated by the interstitial material that

at times they could scarcely be identified. In several places, on the outer half of the media, they had disappeared altogether, with the formation of small, irregularly shaped cysts. The interstitial material stained faintly with hematoxylin and was structureless except that occasional fine fibrils were seen. In some cases the interstitial material was not so plentiful but stained more deeply with hematoxylin as well as with metachromatic dyes. The elastic membranes were widely separated and were as a rule broken and frayed. In the persons whose basal metabolic rate was low before death and in H. H., whose thyroid gland had been removed, calcium deposits were abundant in the muscle layers as well as in the intima.

The nuclei of the muscle cells were normal and interstitial changes were more striking in the inner half of the aortic wall in those persons who had undergone thyroidectomy. The interstitial material stained with basophilic dyes as well as metachromatic stains, polychrome methylene blue, toluidine blue, methylene blue, etc. In the case of H. H., who lived for five years, and in those who had low basal metabolic rates the material took a more acid stain.

In the case of W. B. the intensity of the metachromatic reaction seemed to vary with the size of the accumulation. The larger patches appeared more serous and hence less mucoid. The sudan III and scarlet red stains could be demonstrated in the interstitial chromatrophic material. There was no evidence of an inflammatory reaction or of efforts at repair in any section. In those persons who died shortly after total abolition of their thyroid glands the intima showed no change, although there were fat droplets in the cells underlying the endothelium. In some of the sections from these persons the descending portion of the arch of the aorta showed atheromatous plaques which presented the usual appearance. The adventitia from these aortas was not thickened but contained numerous erythrocytes.

In most of these persons there was moderate thickening of the arterioles in the kidneys and other organs. Numerous glomeruli were fibrotic and hyalinized, but there was some interstitial fibrosis of the kidney, especially of those with long-standing arteriosclerosis. The glomeruli that remained were frequently seen to be congested. The tubular cells frequently showed granular degeneration. The larger vessels had undergone intimal thickening. In many of the smaller arteries the muscle cells of the media showed chromatrophic degeneration similar to that in the coronary arteries and the aorta.

Of the four patients whose thyroid glands had been removed, three had these removed because of heart failure and hypertension and were relatively young. The thyroid gland of the fourth patient had been removed because of emphysema, in an attempt to cut down his oxygen consumption and consequently make his lung function proportioned to

his need of oxygen. All four patients died of cardiovascular disease. The first three patients with hypertension died of spontaneous rupture of the aorta due to idiopathic medial necrosis. The fourth patient, who lived for a period of five years and led a quiet and protected life, died of coronary thrombosis due to a rupture of the coronary artery. This man, who lived for five years after his thyroidectomy, had extremely advanced arterial degeneration. A point of importance in the consideration of this person is that he had emphysema which is believed to be associated with a disturbance of lung activity. Some observers state that arteriosclerosis is an elastic tissue disease, which would bring up the question of whether or not the degenerative changes seen in the aorta might not be due to the same process occurring in the lung. This man gave a history of having had influenza in 1918, and the emphysema was believed to be an aftermath of the infection. There was no clinical or anatomic corroboration of such a point of view.

A study of the City Infirmity Hospital series of cases indicates that persons with low basal metabolic rates as well as athyroid states do have a definite tendency toward degenerative changes in the smooth muscles of the blood vessels, whereas elderly persons who have a normal basal metabolic rate over a period of years before death do not have as advanced arterial changes.

The work of Blumenthal, Lansing and Wheeler³ particularly has indicated the frequent occurrence of medial change in human aortas. These observers showed that degeneration of the media, which may include calcification, is always a primary condition and may be present at a very early age. They found that calcification occurs more frequently than intimal plaques and that intimal plaques do not occur without calcification of the media or some other change, such as syphilitic aortitis or marked connective tissue infiltration of the media. These observers expressed the opinion that medial calcification is more intense in the immediate vicinity of an intimal plaque than elsewhere. They expressed the belief that calcification of the media of the aorta is primarily a function of age and is not influenced by sex or chronic infectious disease. They also expressed the belief that calcification of the media of the aorta is primarily associated with elastic elements since at an early stage calcium is seen deposited in the elastic fibers.

That this process is not limited to the aorta is emphasized by the work of Lansing, Blumenthal and Gray,⁴ who studied, by micro-incineration, tissues from 144 patients, both males and females. They found that there is a progressive metachromatic degenerative change

3. Blumenthal, H. T.; Lansing, A. I., and Wheeler, P. A.: *Am. J. Path.* **20**:665-687, 1944.

4. Lansing, A. I.; Blumenthal, H. T., and Gray, S. H.: *J. Gerontol.* **3**:87-97, 1948.

in the media in which calcium deposit later develops. These authors found that there was an increase in the calcium, which was most prominent in the left coronary artery and which began a decade earlier than in the aorta. They demonstrated that with aging there are progressive splitting and multiplication of the elastic fibers of the internal elastic lamella. They found that there is calcification in the elastic fibers but that with age calcification becomes more intense and has a tendency to extend outward from the elastic tissue. These observers held that arteriosclerosis is a primary product of the media and that there must be degeneration of the media for atherosclerotic plaques to form.

Since hypothyroidism is known to affect the media of the blood vessels and produce, first, metachromatic degeneration, it is possible, if one interprets our work in the light of that of Lansing and his co-workers, that arteriosclerosis may be indicated by hypofunction of this gland or a related phenomenon. The changes that they describe have been observed by us in four persons whose thyroid glands had been removed for therapeutic reasons, and in other persons whose basal metabolic rates were persistently low for years before death. This change has likewise been found by others in at least one case of recognized myxedema.

In our four patients whose thyroid glands had been removed, the degree of metachromatic and arterial degeneration depended somewhat on the duration of the athyroidism. The first two patients lived five months; the third patient lived $2\frac{1}{2}$ years but for the greater part of the two year period she received thyroid U. S. P. Her death occurred seven months after she ceased taking thyroid. All three of these persons had severe hypertension and died of rupture of the aorta. The fourth patient, who had asthma, led a protected life because of his illness. He received some iodine in a cough mixture throughout the period after his operation and lived five years postoperatively. He died with a splitting of the coronary artery which produced coronary occlusion. It is interesting that he showed advanced medial calcification and intimal sclerosis. The degree of arteriosclerosis was more advanced than in any other case in the total series, although the patient was only 54 years of age at death. The two persons whose thyroid glands had been removed five months before death and the one who died of aortic rupture after stopping thyroid treatment did not have gross evidence of calcium deposits in the media. There was a metachromatic degeneration in these patients which in our last case was associated with calcium deposits. It is important to emphasize that the changes found were focal in the media of the blood vessels and did not involve the entire muscle coat, suggesting that the process might be related to the damaging effect of athyroidism on the vasa vasorum.

The onset of medial changes in early life described by Lansing and co-workers would seem to question the possibility that medial disease is related to hypothyroidism. However, one does know that in many persons low metabolism develops after infection, etc., and that later there is return of a fairly normal basal metabolic rate with a presumed increase of thyroid function. It would seem possible, therefore, that various periods of hypofunction of the thyroid gland may exist throughout life and may, by producing medial change, lay the foundation for the advanced degenerative medial changes as well as the atherosclerosis seen at a later period.

The series of autopsies on which the present report is based leads one to the belief that the findings of Lansing and co-workers are of extreme importance. One is not entirely in agreement with their suggestion that there must be medial changes to have primary atherosclerosis in man. One feels that elevated blood cholesterol levels may produce intimal disease and secondary disease of the media, independent of primary medial disease. However, their findings are in general agreement with those presented here, especially those in persons known to have athyroidism.

The studies of Foster and Barr⁸ and Kountz and Hemplemann¹ emphasize the need for better understanding of body metabolism with relation to the activity of the glands of internal secretion, especially with relation to evaluation of arteriosclerosis.

There appear to be at least two independent types of degenerative changes that occur in blood vessels of man with arteriosclerosis. One is primary intimal sclerosis with secondary medial change, which is frequently associated with persons who have elevated blood cholesterol or disturbed fat metabolism but not necessarily a low basal metabolic rate. Since the low metabolism and high cholesterol are commonly associated in hypothyroidism, the two may be found in persons with atherosclerotic changes. Primary intimal change may be a part of, but is not necessarily associated with, hypothyroidism. It may occur in persons with normal thyroid activity or possibly in hyperthyroidism to a moderate degree, according to our experience.

The second change which occurs in arteriosclerosis and is commonly associated with hypothyroidism in animals and man is degeneration of the media. This change is primary and begins as a metachromic degeneration of the media and elastic tissue, with calcium deposition later. The work of Lansing and co-workers, particularly, has been of great value in calling attention to the fact that the medial change may be found at a young age. This does not make its occurrence incompatible with decreased thyroid activity, since one does not know too well the thyroid activity throughout the life span except to say that all studies indicate a variable degree of thyroid function in the persons studied as well as a progressive decline with age.

It would seem that Winternitz, Thomas and Le Compte⁵ have the most likely and tenable approach to the subject of vascular degeneration. The fact that hypothyroidism is known to be a damaging factor to the capillaries suggests that it affects the vasa vasorum, a suggestion which supports the contention that arteriosclerosis associated with hypothyroidism begins in the media. One of the big defects in the attempt to correlate the theories as to the cause of arteriosclerosis with the relationship to hypofunction of the thyroid gland is that most of the theories have been based on experimental work done on animals. Most of the animals have a relatively short span of life, and although basic biologic processes may be studied, observations may never be carried to completeness for evaluation and the findings are not the same in animals as in man.

Just how decreased function of the thyroid gland may affect muscles in general and especially the smooth muscle of the blood vessels is not understood. It is possible that its effect may be on the capillaries, since the media is well supplied with vasa vasorum; thus it could be related to decreased blood supply or to stagnation of the blood in the muscle of the vasa vasorum. On the other hand, it is highly probable, too, that thyroid function plays an important part in the nutrition of the muscles. It may be that reduction of thyroid function in an organism causes a defect to develop in the nutrition of the muscle fibers which may affect the elasticity of these tissues. According to Lansing and co-workers, a degenerative process does occur in the elastic tissues. My studies indicated that advanced degeneration of the elastic tissue is present in the athyroid patients.

My attention is particularly attracted to Hueper's classification⁶ as to the vasotoxic agents. He called attention to the factors causing stagnant anoxia and increased permeability of the relaxed vascular wall through an excessive slowing of the blood flow. He pointed out that hypothyroidism, among other factors, may cause changes which are characterized by medial degeneration. The medial degeneration in our cases is similar to that noted in animals after other endogenous hypotoxic agents have been added.

SUMMARY

An anatomic study of four persons whose thyroid glands had been completely removed for therapeutic reasons and a study of 13 other persons, seven of whom had low rates of oxygen consumption, showed

5. Winternitz, M. C.; Thomas, R. M., and Le Compte, P. M.: *The Biology of Arteriosclerosis*, Springfield, Ill., Charles C Thomas, Publisher, 1938.

6. Hueper, W. C.: *Medicine* **29**:397-442, 1941; *Biol. Symposia* **11**:1-42, 1945.

medial degenerative changes in the aorta and larger blood vessels. In contrast, five of the 13 persons who had normal average basal metabolic rates for a five year period before death and one who had a moderate increase in the rate of oxygen consumption did not show this change. Attention was paid to the thyroid gland at autopsy. In the four patients whose thyroids had been removed no evidence of thyroid tissue was found. In those who had a low basal metabolic rate frank degenerative changes were present in the thyroid, and substantiated the clinical impression of low function of this gland. In each instance in which there was diminished thyroid function, medial arterial changes were noted in the blood vessels. In the younger persons whose thyroid glands had been removed and who had lived for a five month period after the operation, areas of cystic degeneration were noted. Another person, the one who lived for $2\frac{1}{2}$ years and took thyroid for a part of the period, showed advanced degenerative cystic necrosis. The one who lived for five years after thyroidectomy and received no thyroid had advanced arteriosclerosis and medial degeneration.

This work reveals that hypothyroidism and its associated metabolic deficiency in man may lead to advanced degeneration of the blood vessels when present over an extended period.

THROMBOCYTOPENIC PURPURA ASSOCIATED WITH SARCOID GRANULOMAS OF THE SPLEEN

PAUL KUNKEL, M.D.

Chief of Medicine, Veterans Administration Hospital, Newington, Conn.; Assistant Clinical
Professor of Medicine, Yale University School of Medicine

AND

RAYMOND YESNER, M.D.

Chief of Laboratory Service, Veterans Administration Hospital, Newington, Conn.; Assistant
Clinical Professor of Pathology, Yale University School of Medicine
NEWINGTON, CONN.

THROMBOCYTOPENIC purpura occurs predominantly in children and young adults. In only 10 per cent of the cases does it begin past the age of 40. It occurs more frequently in females than in males. A family history of a bleeding tendency is by no means unusual.¹ In the idiopathic type the pathogenesis of the disease has been attributed to (1) excessive destruction of platelets in the spleen—an idea favored by Wiseman, Doan and Wilson² and others—and (2) failure of platelet production through the agency of some splenic substance.³ Although the spleen is commonly involved in sarcoidosis, sarcoid granuloma of the spleen alone probably does not occur. Four patients with sarcoid granuloma of the spleen reported by Kay⁴ were all found to have sarcoid lesions elsewhere. The association of thrombocytopenic purpura and sarcoid of the spleen is rare, seven cases having been reported to date.⁴ Of the four patients who were subjected to splenectomy, three were apparently cured, and one died of hemorrhage in the immediate post-

From the Veterans Administration Hospital, Newington, Conn., and the Department of Medicine, Yale University School of Medicine.

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2. Wiseman, B. K.; Doan, C. A., and Wilson, S. J.: *J. A. M. A.* **115**:8, 1940.

3. Dameshek, W., and Estren, S.: *The Spleen and Hypersplenism*, New York, Grune & Stratton, 1947.

4. (a) Kay, S.: *Am. J. Path.* **26**:427, 1950. (b) Berblinger, W.: *Acta davos.* **5**:1, 1939. (c) Kraus, E. J.: *J. Lab. & Clin. Med.* **28**:140, 1942. (d) Nordland, M.; Ylvisaker, R. S.; Larson, P., and Reiff, R.: *Minnesota Med.* **29**:166, 1946. (e) Jersild, M.: *Acta med. Scandinav.* **97**:322, 1938. (f) Bruschi, M., and Howe, J. S.: *Blood* **5**:478, 1950. (g) Enzer, N.: *Am. J. Path.* **22**:663, 1946. Dameshek and Estren.³

operative period. Of those not undergoing splenectomy, two died and the fate of the third is unknown. The patient whose case of thrombocytopenic purpura associated with sarcoid granulomas of the spleen is reported here was apparently cured by splenectomy.

D. B., a 55 year old white man, a merchant, was admitted to the hospital on Dec. 29, 1949 with a six week history of large ecchymoses on the legs and arms, petechiae about the ankles, persistent bleeding after minor razor cuts while shaving, and oozing from the gums. Two weeks before admission he noticed increasing lethargy, weakness and lightheadedness. Two days prior to admission he noted blurring of vision on looking to the right and a severe, persistent left frontal headache. There was no history of familial bleeding, of an occupational hazard or of drug ingestion. The system review was noncontributory.

Physical examination revealed a hypersthenic, well developed and well nourished, but lethargic, white man with generalized ecchymoses over the trunk and extremities and numerous petechiae over the ankles and the wrists. There were crusted blood in the nares and the mouth, oozing of the gums and marked halitosis. There was no glandular enlargement. There was a small conjunctival hemorrhage on the left, and examination of the visual fields revealed a right homonymous hemianopsia. Retinal examination was unrevealing. The liver and the spleen were not palpable.

Laboratory data were as follows: The hemoglobin was 14.0 Gm. The white blood cell count was 5,500 per cubic millimeter, with a normal differential count. No platelets were seen in a smear of the peripheral blood. There was no clot retraction after 24 hours. Biopsy of the bone marrow revealed an abundance of megakaryocytes, but there were no platelets about their periphery. Urinalysis revealed no abnormalities. Lumbar puncture showed an initial spinal fluid pressure of 200 mm. of water and frankly bloody fluid, which when centrifuged yielded a red cell layer beneath xanthochromic fluid.

Splenectomy was performed 24 hours after admission, on Dec. 30, 1949, by Dr. Alfred Hurwitz. Near the lower pole of the spleen in the great omentum an accessory spleen was identified and removed. The pancreas was palpated and seemed slightly larger than normal. The surgical procedure was deemed urgent to obviate further intracranial hemorrhage.

One hour after operation a smear of the peripheral blood revealed the presence of rare giant platelets, but normal platelets were not seen. Twelve hours after operation a smear revealed platelets in every field with abundant giant forms. At 48 hours there were 300,000 platelets per cubic millimeter of blood. The platelet count on the fourth day was 129,270, that on the fifth day 130,510 and that on the seventh day 118,000.

Further immediate postoperative studies included first and second strength intradermal tuberculin tests made with purified protein derivative, U. S. P., the results of which were negative, liver function tests, which showed normal functioning, and determinations of blood calcium (normal), total serum protein (7.3 Gm. per 100 cc., with an albumin-globulin ratio of 1.2), bleeding time (2½ minutes), clotting time (4½ minutes) and clot retraction (prompt). A roentgenogram of the chest revealed no abnormality. During the next six weeks the platelet count dropped slowly to 22,000 per cubic millimeter, and during the sixth week petechiae were again noted about the ankles. The petechiae disappeared suddenly, and by the tenth week the platelets had risen to 430,000, the spinal fluid had cleared and there was remarkable improvement in the visual field defect and in reading ability. The patient was discharged on the sixty-first postoperative day.

When the patient was reexamined 12 weeks postoperatively, the findings were within normal limits except that the roentgenogram of the chest revealed for

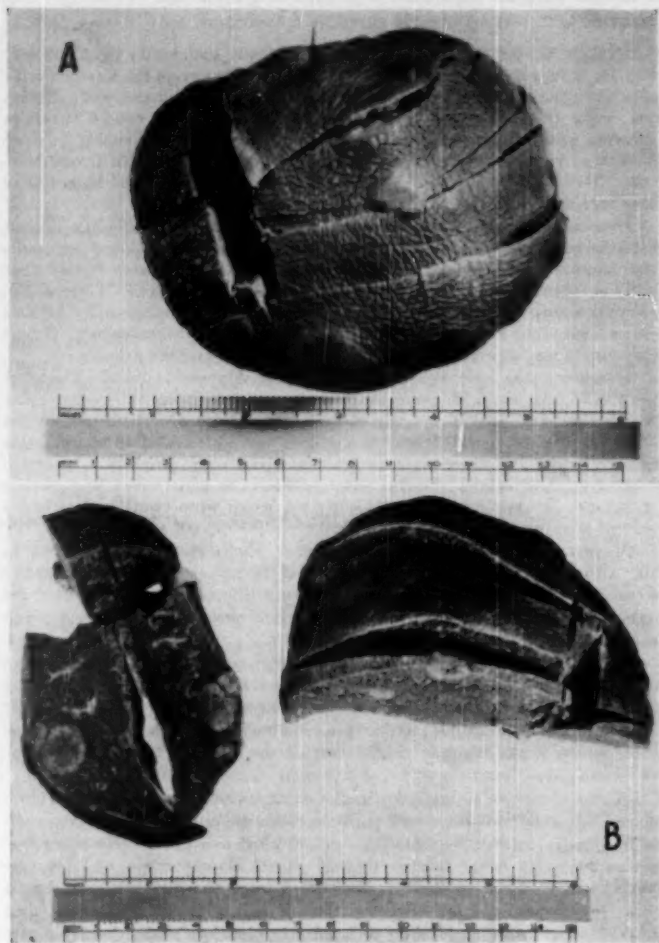


Fig. 1.—Gross spleen. *A* shows the wrinkled capsule and the rounded nodular surface elevations; *B*, the cut surfaces with sharply demarcated light nodules.

the first time a diffuse granularity throughout both lung fields consistent with a diagnosis of diffuse sarcoidosis.

The patient received 1,100 cc. of fresh whole blood during the surgical procedure, and on the one hundred and fiftieth postoperative day jaundice developed and he was readmitted. A diagnosis of homologous serum jaundice, confirmed by liver biopsy, was made. After nine weeks of hospitalization liver function returned to normal, the bleeding and clotting times and the platelet count remained normal and the chest roentgenograms remained unchanged. He was discharged asymptomatic and has remained well to date.

Pathological Observations.—The spleen weighed 210 Gm. After its blood was allowed to ooze from the resected pedicle, its weight was reduced to 190 Gm. The capsule was smooth, thin and gray and was elevated in several places by rounded subcapsular nodules. These projected 2 mm. above the general surface of the spleen and had the appearance of metastatic tumor (fig. 1A). After the loss of blood, the capsule of the spleen became generally wrinkled except over these nodules, where it remained relatively smooth. The spleen was of firm consistency and deep red. Numerous nodules measuring from 8 mm. to 2.5 cm. were found throughout the organ. The red pulp, on section, retracted from the light yellow nodules, causing them to bulge above the cut surface and bringing them into sharp relief (fig. 1B). The splenic pulp was otherwise of normal appearance and consistency. The malpighian bodies were not grossly enlarged. Accompanying the spleen was a small accessory spleen, measuring 1 cm. by 8 mm. by 8 mm. It was covered by a thin capsule and was of a dark purple-red color. The cut surface appeared to be moderately fibrous, of a lighter red color, and presented no nodules like those seen in the spleen proper.

Microscopic Examination.—The spleen was studded with numerous epithelioid granulomas, varying from a few epithelioid cells forming an irregular mass in a malpighian body to large, lobulated masses with rather loosely arranged cells and encapsulated by laminated, hyalinized collagenous envelopes. Hyaline strands frequently entered the lobulated masses of granulomas, further subdividing them. Numerous giant cells were present in the granulomas. They tended to have abundant cytoplasm and numerous small vesicular nuclei grouped around the periphery of the cell. No Schaumann or asteroid bodies were demonstrated. Many of the nodules had at the center a well formed small blood vessel (fig. 2A). There was no evidence of necrosis to suggest tuberculosis, and no parasitic organisms were found. Acid-fast stains gave negative results. The splenic tissue about many of the nodules was filled with fresh blood (fig. 2B). The small blood vessels of the spleen showed hyaline beading of the arteriolar walls. The sinusoids of the spleen tended to be quite empty of red cells and contained an occasional megakaryocyte (fig. 3). The splenic pulp contained an occasional eosinophilic cell and a few polymorphonuclear granulocytes and lymphocytes. No phagocytosis of platelets or other blood elements was demonstrated. The malpighian bodies were of normal size and devoid of germinal centers.

Bone Marrow Studies.—The first marrow aspiration was done on the day before operation (fig. 4A). The marrow contained 74 per cent cells of the granulocytic series, 21 per cent lymphocytes, 3 per cent monocytes, 1 per cent eosinophilic cells and 1 per cent plasmacytes. There were 35 nucleated red cells per hundred white blood cells. Megakaryocytes were numerous, four to five being present per high power field. Twelve hundred and ninety megakaryocytes were enumerated per million nucleated red cells, according to the method of Dameshek and Miller.⁵ Numerous young forms, particularly promegakaryocytes, were present, as well

5. Dameshek, W., and Miller, E. B.: *Blood* 1:27, 1946.

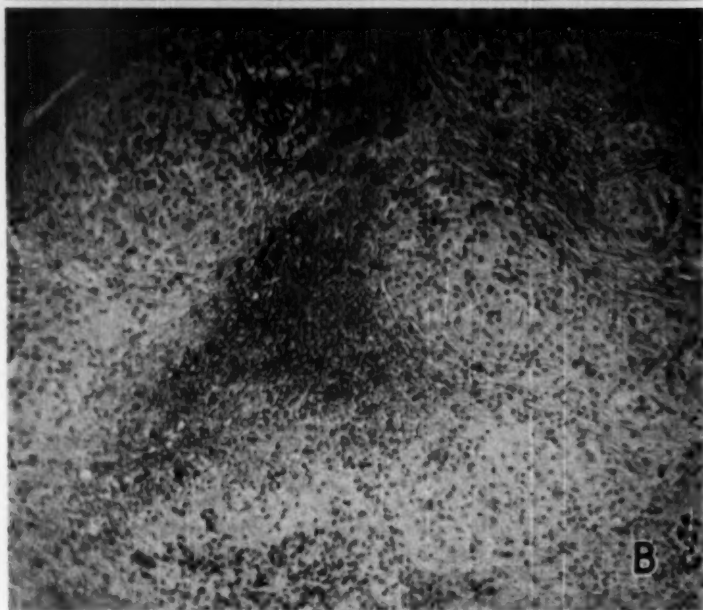
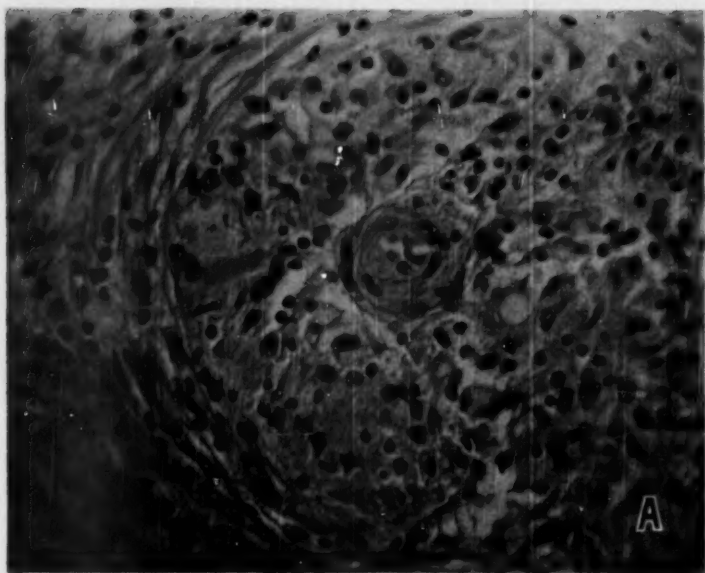


Fig. 2.—*A*, sarcoid granuloma with central blood vessel and sharply outlined periphery (photomicrograph, oil immersion objective). *B*, sarcoid granulomas, encapsulated by fibrous tissue. Hemorrhage is seen in the center of the field (photomicrograph, $\times 150$).

as adult forms. The cytoplasm of these cells was vacuolated and deficient in granules. No platelet production was found, nor were platelets present in the marrow or in the accompanying peripheral blood smears. The second marrow aspiration was done on Jan. 16, 1950 (fig. 4B). There were 67 per cent cells of the granulocytic series, 25 per cent lymphocytes, 3 per cent monocytes, 3 per cent eosinophilic cells, 2 per cent plasmacytes and 19 nucleated red cells per hundred white cells. The megakaryocyte count was 1,220 per million nucleated red cells. The megakaryocytes were nearly all adult forms with multilobulated nuclei and well granulated cytoplasm, and platelets were present at their periphery. The third specimen of marrow was aspirated on Feb. 16, 1950, 48 hours after a recurrence of ecchymoses and petechiae. There were 72 per cent cells of the granulocytic

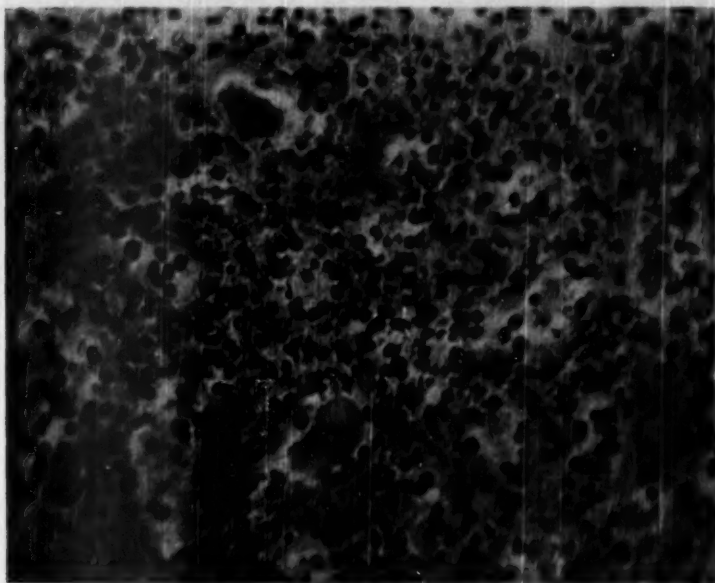


Fig. 3.—Splenic pulp showing relatively empty sinusoids and two megakaryocytes.

series, 25 per cent lymphocytes, 2 per cent monocytes, no eosinophilic cells, 1 per cent plasmacytes and 52 nucleated red cells per hundred white cells. The megakaryocytes appeared normal and were markedly abundant. The megakaryocyte count was 1,570 per million nucleated red cells. The platelets in the marrow smears and in the associated peripheral blood smears were sharply reduced. A fourth aspiration of marrow was performed on May 18, 1950 because of possible serum hepatitis. The white cell count was 9,300 per cubic millimeter, with 58 per cent lymphocytes. The red cell count was 4,280,000, and there were 259,000 platelets per cubic millimeter, with a negative tourniquet test. There were 38 per cent cells of the granulocytic series, 53 per cent lymphocytes, 7 per cent monocytes,

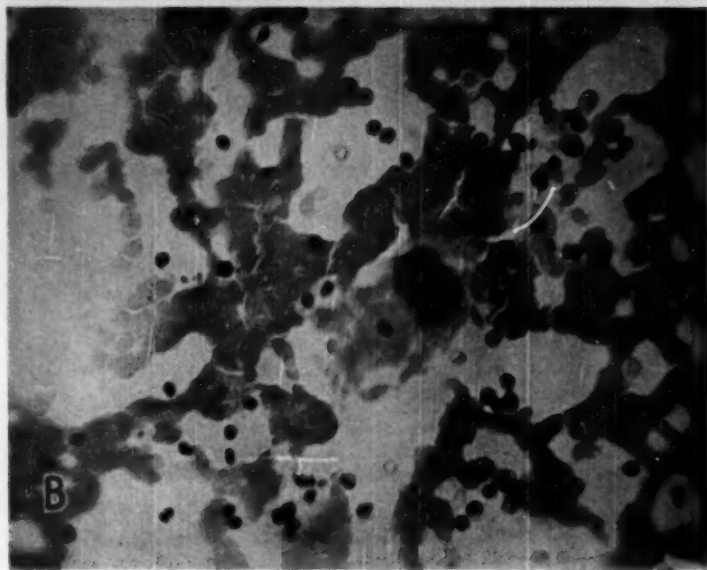
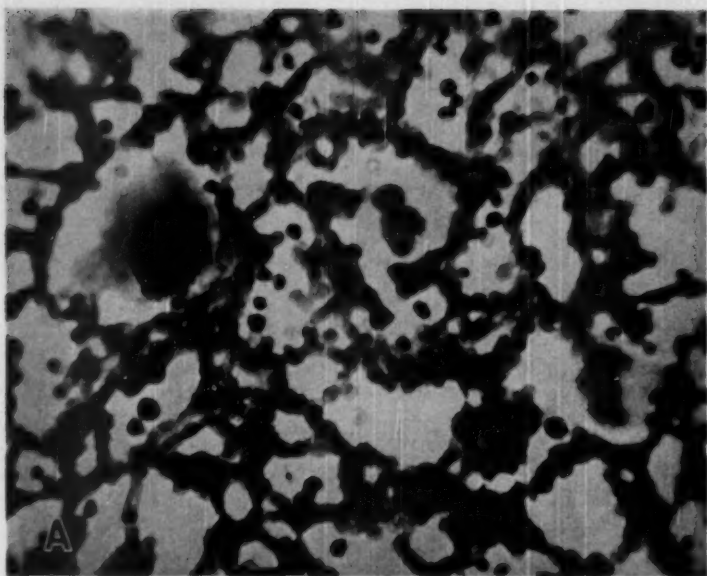


Fig. 4.—A, megakaryocytes in bone marrow, Dec. 30, 1949. The larger cell has a compact, nonsegmented nucleus (photomicrograph, oil immersion objective). B, megakaryocyte in bone marrow, Jan. 16, 1950. The nucleus is elongated and folded. Platelets are attached to the periphery of the cell (photomicrograph, oil immersion objective).

2 per cent eosinophilic cells and 14 normoblasts per hundred white cells. The megakaryocyte count was 660 per million nucleated red cells. The megakaryocytes were of adult form and normal appearance. Biopsy of the liver was performed on May 19, 1950 because of mild jaundice and hepatomegaly occurring 150 days after the latest blood transfusion. Alkaline phosphatase was $13\frac{1}{2}$ units, and a test of urine for urobilinogen was positive in the dilution of 1 to 100. The material secured by needle biopsy showed the characteristic picture of infectious hepatitis with necrotic liver cells, round cell infiltration of the lobules, focal collections of chronic inflammatory cells and bile pigmentation of the Kupffer cells. There were no sarcoid lesions in the liver sections.

COMMENT

The case presented exhibited the cardinal signs and symptoms of idiopathic thrombocytopenic purpura. There were ecchymoses, petechiae and hemorrhage involving the central nervous system, absence of platelets in the peripheral blood smear, lack of clot retraction and marked hyperplasia of the megakaryocytes in the bone marrow with failure of platelet production.

The skin test made with purified protein derivative of tuberculin U. S. P. provoked no reaction, and on reexamination the chest roentgenogram revealed diffuse granularity throughout both lung fields, consistent with sarcoidosis of the lungs.

The continued granularity of the lung fields indicates that the sarcoidosis persists. The clinical course, the peripheral blood smears and the results of the bone marrow studies suggest that the purpura has been cured.

Statistically it is probable that thrombocytopenic purpura associated with, or mediated by, sarcoid granulomas is more common than reports would indicate. The evidence that in this case it was of the idiopathic variety is meager inasmuch as (1) the patient was a male, (2) he was considerably older than the age group usually involved and (3) there was a lack of the characteristic eosinophilic cell infiltration and of the prominent germinal centers in the malpighian bodies of the spleen.

The pathological observations indicate that the spleen was not hyperactive in the phagocytosis of platelets but rather that an arrest of maturation occurred in the bone marrow associated with megakaryocytic hyperplasia, lack of granularity, vacuolation and failure of platelet production. It is possible that the sarcoid may have mediated the production of the same agent responsible for failure of platelet production as observed by Frank⁶ and Dameshek and Miller⁶ in the idiopathic form of thrombocytopenic purpura. It has been suggested that reticuloendothelial hyperplasia of whatever cause is the source of the hypothetical marrow depressant resulting in maturation arrest.

6. Frank, E.: *Berl. klin. Wchnschr.* 52:454, 490, 1915.

Such hyperplasia did not occur in the sections of the spleen and bone marrow which were examined except in the granulomas themselves. In Enzer's case ²⁸ of pancytopenia with purpura and sarcoid granulomas of the spleen, no lesions were found in the marrow at autopsy.

Furthermore, it is most unlikely that splenectomy would remove enough of the reticuloendothelial system to produce a cure if this mechanism were responsible.

In five of the cases of thrombocytopenic purpura associated with splenic sarcoid reported to date, the patients were subjected to splenectomy, with four cured.

SUMMARY

A case of thrombocytopenic purpura associated with sarcoid is reported. Evidence is presented to indicate that the thrombocytopenia was due to arrest of the maturation of the megakaryocytes of the bone marrow, probably related to the splenic agent said to occur in idiopathic thrombocytopenic purpura.

STUDIES ON THE ACUTE TOXIC EFFECTS OF 4-AMINO-PTEROYLGLUTAMIC ACID IN DOGS, GUINEA PIGS AND RABBITS

Difference in Species Susceptibility and Protective Action of Folic Acid

VIRGINIA MINNICH, M.S.

CARL V. MOORE, M.D.

DAVID E. SMITH, M.D.

AND

GLADDEN V. ELLIOTT, M.D.

ST. LOUIS

AMINOPTERIN, or 4-aminopteroylglutamic acid, is the most potent known antagonist of pteroylglutamic, or folic, acid. It has been extensively used in investigations designed to determine the physiological interrelationships of folic acid,¹ in studies which evaluated its effect on animals and in the treatment of acute leukemia in man.² An earlier report from this laboratory described the hematological changes produced in guinea pigs by the administration of relatively small doses of the antagonist given over an extended period.³ The present report defines the acute toxic effects produced by large doses of 4-aminopteroylglutamic acid in dogs, guinea pigs and rabbits, calls attention to a striking difference in species susceptibility to 4-aminopteroylglutamic acid and presents evidence to indicate that very large amounts of folic acid can protect dogs, at least, from doses of the antagonist which otherwise are lethal.

From the Departments of Internal Medicine and Pathology, Washington University, School of Medicine.

This investigation was supported in part by research grant H-22 from the United States Public Health Service, and in part by a grant from the Lederle Laboratories Division of the American Cyanamid Company.

1. Nichol, C. A., and Welch, A. D.: *Proc. Soc. Exper. Biol. & Med.* **74**:403, 1950.

2. Reinhard, E. H.; Good, J. T., and Martin, E.: *J. A. M. A.* **142**:383, 1950.

3. Innes, J.; Innes, E. M., and Moore, C. V.: *J. Lab. & Clin. Med.* **34**:883, 1949.

MATERIAL AND METHODS

Three species of animals were used for the experiments: (a) healthy mongrel dogs, (b) young guinea pigs weighing 250 to 550 Gm. and (c) young adult rabbits weighing 1.5 to 2 Kg. Each animal was housed in an individual cage and given food ad libitum. The dogs were fed a commercial dog food^{4a}; the other animals received a commercial rabbit chow.^{4b} Lettuce was also regularly provided for the guinea pigs. No attempt was made to influence synthesis of pteroylglutamic acid on the part of intestinal organisms by including any of the sulfonamide compounds in the diet.

Solutions of 4-aminopteroylglutamic acid⁵ were prepared by dissolving a weighed amount of the substance together with the same weight of sodium bicarbonate in sufficient distilled water to yield final concentrations of 5 and 10 mg. per cubic centimeter. They were then autoclaved. When necessary, these solutions were diluted with sterile distilled water in order to facilitate accurate measuring of smaller doses. Injections of the material were made into the subcutaneous tissue of the neck.

In each experiment, the normal range of peripheral blood values was established during a control period of two to seven days. Blood was obtained for counts by puncturing an ear vein with a Bard-Parker scalpel blade. Throughout the period of observation, frequent determinations were made of the red blood cells per cubic millimeter, the hemoglobin,⁶ the total white blood cells per cubic millimeter, the differential percentages, the reticulocyte percentage and the thrombocyte level.⁷ Both supravital and fixed films stained with Wright's stain were employed. Bone marrow was aspirated from the iliac crest during life or obtained from the head of the femur at autopsy. Postmortem examination was made as soon as possible after the animal died; blocks of tissue were taken for histological examination from the following organs: heart, liver, spleen, kidneys, adrenal glands, gastrointestinal tract and bone marrow.

EXPERIMENTAL OBSERVATIONS

Changes Induced by 4-Aminopteroylglutamic Acid and Differences in Degree to Which Dogs, Guinea Pigs and Rabbits Were Affected by the Antagonist.—Early in the course of these experiments it became evident that there was a significant difference in the response of these three species of animals given injections of 4-aminopteroylglutamic acid. Dogs were much more susceptible to toxic effects than were guinea pigs, and guinea pigs seemed slightly more susceptible than rabbits.

1. Effects on Dogs: In experiments on seven dogs, 4-aminopteroylglutamic acid was injected subcutaneously in daily doses of 0.25 to 5 mg. for from five to eight days (table 1). This amount provided 0.023 to 0.48 mg. per kilogram of body weight. Four of the dogs died on the sixth to the ninth day of the experiment.

4. (a) The food used is marketed under the name of "friskies dog food cubes"⁸ by Albers Milling Company, a division of the Carnation Company, Peoria, Ill. (b) The chow used is marketed under the name of the "complete rabbit chow" by Ralston Purina Company, St. Louis.

5. The 4-aminopteroylglutamic acid and the pteroylglutamic acid used in these experiments were supplied by Dr. Guy Clark, of the Lederle Laboratories, Inc.

6. Evelyn, K. A.: J. Biol. Chem. **115**:63, 1936.

7. Dameshek, W.: Arch. Int. Med. **50**:579, 1932.

Whenever the dose was greater than 0.04 mg. per kilogram, the animals began to eat less on the third or fourth day of injections, vomited frequently and lost weight rapidly. A severe degree of glossitis developed, and bleeding of the gums occurred. These changes were soon followed by bloody diarrhea, so severe that blood dripped from the rectum. The resultant dehydration was associated with a rise of the red cell and hemoglobin levels.

The most consistent changes in the peripheral blood were decreases in reticulocytes and lymphocytes. Total eosinophil counts were not done, but there was an unmistakable tendency for these cells to become less numerous. With the two highest doses (dogs 1 and 2) severe leukocytopenia developed rapidly. In five

TABLE 1.—Effect of 4-Aminopteroylglutamic Acid on Peripheral Blood of Dogs

Dog	Daily Dose Injected		Day of Expt.	Wt., Kg.	RBC $\times 10^6$ per Cu. Mm.			WBC per Cu. Mm.		Lymphocytes per Cu. Mm.	Reticulocytes, %	Platelets $\times 10^6$ per Cu. Mm.		Survival, Days
	Total, Mg.	Mg. per Kg.*												
1	5	.49	0	10.5	7.90	16.3	5,600	2,100	3.0	800				
	(for 5 days)		5	9.0	8.48	20.4	1,700	480	0.3	3,540				6
2	2.5	.29	0	4.4	7.90	17.2	11,000	2,700	1.8	1,610				
	(for 5 days)		0	5.0	9.08	20.7	1,900	500	0	4,000				7
5	0.5	.06	0	10.5	6.60	15.2	20,850	1,600	2.6	2,490				
	(for 5 days)		5				
	1.0	0.1	6	10.0	6.48	16.7	16,000	900	1.6				
	(for 5 days)		10	8.2	8.11	19.2	9,300	270	1.0	1,185				
			18	8.4	6.18	16.6	5,800	410	0	1,240				
			21	9.3	4.61	12.5	27,600	2,480	4.6	1,180				
			45	11.4	6.32	17.7	14,600	1,130	8.2	2,700				Survived
4	0.5	.04	0	12.3	7.44	18.7	12,400	2,000	0.8	1,808				
(a)	(for 6 days)		5	9.3	5.60	23	10,600	580	0.2	2,228				
			14	8.4	3.65	11	39,700	704	0	600				
			18	8.2	3.00	8	62,600	1,900	2.5	900				
			20	8.4	2.82	7.7	22,600	1,625	28.1	808				
			45	8.6	5.00	14.5	25,000	2,300	3.0	1,900				Survived
	(interval of 11 months)													
(b)	0.5	.04	0	12.3	7.82	17.8	15,500	2,110	0.8	1,720				
	(for 6 days)		17	12.0	7.80	17.8	16,200	2,800	0.2	2,475				
			22	12.5	8.10	18.0	22,300	4,014	2.0	2,673				Survived
3	0.25	.041	0	6.0	5.73	16.5	10,500	1,950	0.2	570				
	(for 6 days)		8	4.8	7.12	19.8	27,000	270	0	613				9
6	0.25	.08	0	8.4	7.35	17.9	9,300	1,690	0.2	1,800				
	(for 5 days)		5	8.4	7.12	19.5	7,600	394	0.4				
	1.0	.15	8	7.6	10.06	24.5	18,300	185	0	2,090				9
	(for 3 days)					
7	0.25	.065	0	11.0	6.39	18.0	14,000	2,550	1.4	1,440				
	(for 7 days)		7	10.0	7.96	18.2	10,800	1,150	4.0	2,900				
			20	11.4	7.38	18.2	20,800	3,380	1.3	1,640				Survived

* This was calculated on the basis of the weight at the beginning of the experiment.

instances the thrombocyte level rose after hemorrhage began; thrombocytopenia was never observed. There was a striking decrease in the number of nucleated red blood cells in the bone marrow without any detected shift to younger forms. No cells resembling megaloblasts were seen. In the case of dogs 1, 2 and 3 bone marrow was obtained from ribs, vertebrae and the head of the femur at autopsy. All sections demonstrated advanced hypoplasia with fat replacement. There were slight congestion of capillaries and engorgement of sinusoids.

Dog 5 and during period "a" dog 4 were particularly interesting. These animals became extremely ill but survived long enough to undergo spontaneous remissions. Dog 4 became semicomatose on the eighth day and was so dehydrated that he was given 250 cc. of 5 per cent dextrose intravenously on two successive days. After five or six days of continued anorexia, gastrointestinal bleeding, severe reticulocytopenia and a falling red cell count, recovery took place. Reticulocytes reappeared on about the eighteenth day and nucleated erythrocytes increased

in the marrow. A few moderately young erythroblasts were observed, but no megaloblasts. In dog 4 the reticulocyte peak reached 28 per cent. At about the same time, a transient granulocytic leukocytosis developed; as many as 10 per cent of the cells were myelocytes and 25 per cent metamyelocytes, but there was also a return of lymphocytes to control levels. The red cells gradually rose, gastrointestinal manifestations subsided, weight stabilized or increased and the animals again became vigorous and normal in appearance.

An attempt was made to determine whether in these two animals the anemia was caused solely by a hemolytic action of the drug. The hemorrhagic diarrhea, however, was so severe that feces could not be collected in quantity for urobilin determination. Serum bilirubin levels, measured in dogs 1 to 4, showed only negligible increases (control values, 0.05 to 0.13 mg. per 100 cc.; maximum values, 0.18 to 0.29 mg. per 100 cc.).

Necropsy revealed congestion of the gastrointestinal tract, with petechiae visible on the mucosal surface, particularly near the rectum. Hemorrhage and

TABLE 2.—Effect of 4-Aminopteroylglutamic Acid on Peripheral Blood of Guinea Pigs

Guinea Pig	Daily Dose Injected			Day of Exper.	Wt., Gm. [†]	RBC × 10 ⁶		Hb., Gm.	WBC per Cu. Mm.		Lymphocytes per Cu. Mm.	Reticu- loeytes, %	Platelets × 10 ³		Survival, Days
	Total, Mg.	Mg. per Kg.*	per Cu. Mm.			per Cu. Mm.	per Cu. Mm.		per Cu. Mm.						
4	5.0	...	0	...	6.35	17.2	...	9,450	6,150	2.0	2,430				
			12	1.51	4.0	...	3,625	2,500	0	23	13			
5	2.5	...	0	5.73	14.1	...	7,500	4,800	1.1	1,983				
			11	4.92	13.9	...	1,300	1,080	0	98	12			
6	2.5	...	0	6.04	15.9	...	10,700	6,550	2.2	1,807				
			12	3.92	10.5	...	2,500	2,120	0	51	13			
8	1.0	...	0	6.20	14.5	...	9,800	6,400	2.2	1,540				
			12	4.81	14.0	...	750	570	0.2	953	12			
10	1.0	1.96	0	410	5.77	14.2	...	7,230	4,000	2.6	1,280				
	(for 28 days)		28	475	2.01	4.0	...	4,100	1,800	0.8	1,000				
			32	435	1.67	4.6	...	9,100	5,800	34.9	574				
			35	355	3.30	15.9	...	21,000	5,900	1.2	1,773				
			77	400	6.45	14.5	...	15,700	7,800	0.6	2,670	Survived			
14	0.5	1.11	0	450	5.39	14.6	...	9,750	5,800	2.6	2,100				
	(for 20 days)		21	310	2.15	5.8	...	5,100	1,800	0.8	997	23			
18	0.5	1.72	0	290	5.63	14.5	...	12,000	4,100	2.6	2,100				
	(for 21 days)		21	278	5.07	7.9	...	4,900	2,500	1.2	1,248				
			26	298	3.30	9.2	...	24,500	3,400	7.2	2,400	26			

* This was calculated on the basis of the weight at the beginning of the experiment.

† The weights of guinea pigs 4, 5, 6 and 8 were not obtained, but they were relatively small.

focal areas of atelectasis were found in the lungs of dog 3. No other gross pathological changes were observed. Microscopic examination of sections from the gastrointestinal tract showed great engorgement of the mucosal vessels of the stomach with hemorrhages in the tips of the villi of the lamina propria, congestion of the intestinal mucosa and degenerative epithelial changes (dogs 1 and 2) of the type described by other workers.⁸ There was advanced congestion of the liver with slight atrophy of the central hepatic cords. Advanced congestion, an increase in reticulum cells and open sinusoids were noted in the spleen, together with hemorrhages around a few of the trabeculae and a decrease in the number and the size of follicles, a few of which had active centers.

2. Effects on Guinea Pigs: Seven guinea pigs were given an injection of 0.5 to 5 mg. of 4-aminopteroylglutamic acid each day for eleven to 28 days (table 2). Unfortunately, the weights of animals 4, 5, 6 and 8 were not obtained, but it is

8. Rinehart, J. F., and Greenberg, J. D.: *Am. J. Path.* 24:710, 1948. Wall, E.: *Arch. Path.* 46:559, 1948.

known that they were relatively small. Even if it is assumed that these guinea pigs weighed 500 Gm. each, the daily dose provided from 1.1 to 10 mg. per kilogram, amounts which are approximately 20 or more times greater than those given each day to the dogs. The injections, furthermore, were continued for a longer period of time. In spite of these factors, toxic manifestations were less severe, and survival times were longer (twelve to 26 days). Guinea pig 10, given 2.1 mg. per kilogram of body weight for 28 days, survived the administration. All the animals lost weight. No lesions appeared in the oral cavity. Bloody diarrhea was observed terminally in several instances but, except in guinea pig 14, was always associated with thrombocytopenia.

The severity of the hematic changes varied with the size of the individual dose and the duration of administration. Reticulocytopenia occurred early in all instances and was followed by the development of a normocytic normochromic anemia of variable severity. In guinea pig 4, given 5 mg. each day, the red cell count fell to 1.51 million cells on the thirteenth day. In animals 10 and 14 (1.0 and 0.5 mg. daily) the level decreased to 1.67 and 2.15 million cells on the thirty-second and twenty-first days, respectively. Granulocytopenia and concomitant lymphocytopenia appeared uniformly. Terminally, in guinea pigs 5, 6 and 8 lymphocytes constituted 75 to 94 per cent of all white cells. Granulocytes showed hypersegmentation of their nuclei, with some cells containing as many as 14 lobes. There was no consistent tendency for the percentage of eosinophils to decrease. Thrombocyte levels fell precipitously in those animals which received 2.5 to 5 mg. per day (nos. 4, 5 and 6) but did not drop in the other instances.

The bone marrow of all animals showed a definite diminution in erythroid cells; a few young erythroblasts could be found, but one had to search for them. The hypocellularity extended to granulocytic elements, and in those animals given the larger doses (nos. 4, 5, 6 and 8) the hypoplasia was advanced. Karyorrhexis of the nuclei was present. Megakaryocytes were markedly reduced in animals 4 and 5. Large cells of the type previously called "reticulum cell"² were increased in number: guinea pigs 14 and 18 died three and five days, respectively, after injections of 4-aminopteroylglutamic acid had been discontinued. In the marrow specimens obtained from the two animals at autopsy, beginning recovery was apparent; the marrow was cellular and definitely hyperplastic for nucleated erythrocytes. No evidences of maturation arrest were found.

The observations on guinea pig 10 are of particular interest because this animal recovered from the effects of 4-aminopteroylglutamic acid after the antagonist had been given in a daily dose of 1 mg. for 28 days (chart 1). Four days after the last injection, a striking reticulocytosis of 34.9 per cent appeared; a rise in red cells followed shortly thereafter.

Macroscopic examination of tissues at the time of death revealed few significant changes. There were areas of hemorrhage in the intestinal wall of guinea pig 14. Splenomegaly was present in guinea pigs 14 and 18; hepatomegaly, in guinea pig 14.

Fatty metamorphosis was the most constant histological abnormality noted in the liver. Hemosiderin granules were found in the spleen; follicles were normal, without reactive centers. Reticulum hyperplasia was noted in the spleens of guinea pigs 14 and 18. Congestion of the spleen with prominent sinusoids and diffuse fibrosis was observed in guinea pig 14. In none of the lymph nodes examined was there evidence of active proliferation or destruction of cells; the general appearance was of inactivity or slight hypoplasia. Adrenals from four animals were examined and showed no histological lesions.

3. Effects of 4-Aminopteroylglutamic Acid in Rabbits: Four rabbits were given 5 or 10 mg. of 4-aminopteroylglutamic acid per day for nine to 33 days.

These doses were equivalent to 2.5 to 6.2 mg. per kilogram of body weight (table 3). The animal which received the largest amount of antagonist lost weight, had diarrhea terminally but otherwise appeared normal up to the time of death on the thirty-third day of administration. Rabbits 2 and 3 survived for only nine and 13 days; a watery diarrhea developed during the terminal four or five days in both animals. Rabbit 4 was killed by a dog which had broken out of its cage; at the time of death, on the seventeenth day of administration, this rabbit had lost no weight and had shown no evidence of toxicity.

Hematologic variations from the normal were much less striking than in the guinea pigs. Erythrocyte, hemoglobin and thrombocyte levels did not change significantly in any instance. Reticulocytopenia occurred only in rabbit 3. The white blood cells fell to 1,250 per cubic millimeter in rabbit 1. Lymphocytopenia was

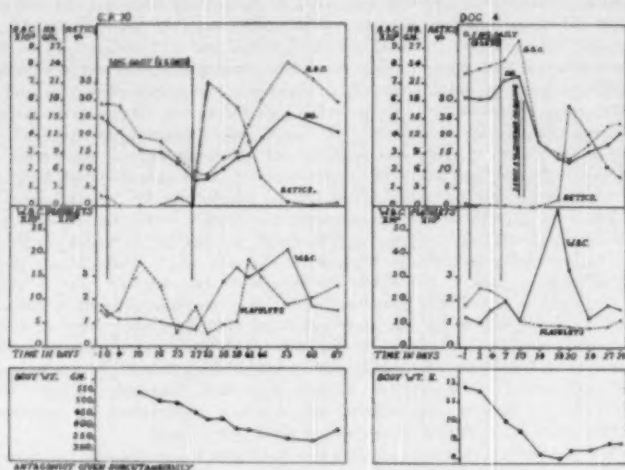


Chart 1.—Data on guinea pig 10 and dog 4, which recovered from anemia produced by 4-aminopteroylglutamic acid, antagonist of folic acid.

TABLE 3.—Effect of 4-Aminopteroylglutamic Acid on Peripheral Blood of Rabbits

Rabbit	Daily Dose Injected		Day of Exper.	Wt., Kg.	RBC $\times 10^6$ per Cu. Mm.	Hb., Gm.	WBC per Cu. Mm.	Lymphocytes per Cu. Mm.	Reticulocytes, %	Platelets $\times 10^6$ per Cu. Mm.	Survival, Days
	Total, Mg.	Mg. per Kg.*									
1	10	6.2	0	1.7	5.38	12.0	8,150	2,000	3.6	1,124	23
			23	1.3	4.75	10.5	1,250	895	2.0	1,847	
2	5	2.7	0	1.9	5.40	12.0	6,750	4,800	5.6	3,128	9
			9	1.7	6.01	12.4	18,500	1,100	5.6	2,496	
3	5	2.8	0	1.8	5.30	12.6	7,550	3,560	6.0	2,078	13
			13	1.5	4.76	12.3	4,900	1,120	0.9	1,700	
4	5	2.5	0	2.0	5.41	13.7	11,500	5,460	1.4	1,580	17 (killed)
			17	2.0	5.15	12.6	31,000	23,700	3.8	1,530	

* This was calculated on the basis of the weight at the beginning of the experiment.

observed in three of the animals. Leukocytosis developed in rabbits 2 and 4. The percentage of eosinophils did not decrease.

No gross abnormalities were observed at necropsy. Histological examination of the lungs showed edema and congestion. The spleens of rabbits 1 and 3 were congested and had large follicles with active germinal centers. Reticulum cells, between small follicles without germinal centers, were prominent in the spleen of rabbit 4; there was no congestion. The bone marrow was hypoplastic, congested and contained the granular basophilic debris characteristic of an acutely hypoplastic marrow. The hypoplasia involved all marrow elements; the ratio of white cells to nucleated erythrocytes was not altered. In the preparations from rabbit 4, areas of normal cellularity were scattered through the predominantly hypoplastic marrow. No megaloblast-like cells were observed. "Reticulum cells" similar to those found in guinea pig marrow were present in small numbers.

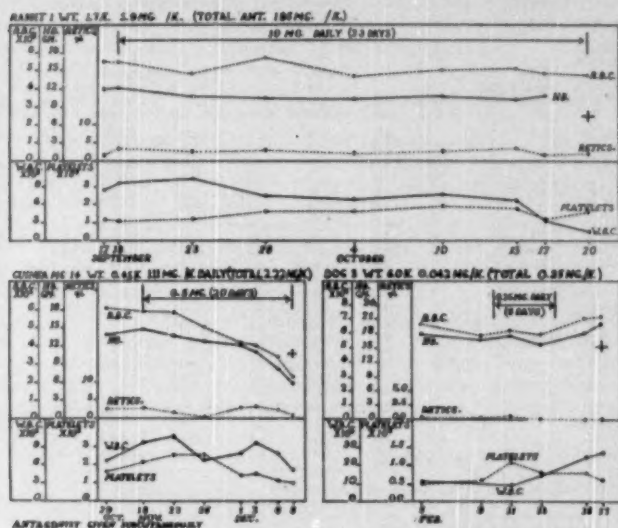


Chart 2.—Data on rabbit 1, guinea pig 14 and dog 3 showing species difference in susceptibility to the action of 4-aminopteroylglutamic acid.

Even though the observations described in the foregoing sections were made on small numbers of animals, they demonstrate clearly that (1) the rather large doses of 4-aminopteroylglutamic acid employed cause hypoplasia of all marrow elements, (2) nucleated erythrocytes are the first cells to disappear from the marrow of dogs and guinea pigs, (3) a species difference appears in the degree to which animals are susceptible to the toxic effects of the antagonist and (4) marrow may recover spontaneously from the depressant effects of 4-aminopteroylglutamic acid. The data in chart 2 emphasize the species difference. No evidences of maturation arrest were detected in the bone marrow.

Effect of Pteroylglutamic Acid and of Purified Liver Extract on the Toxic Manifestations Produced by 4-Aminopteroylglutamic Acid.—Large doses of pteroylglutamic acid (folic acid) and/or of purified liver extract failed to prevent the death of guinea pigs given 4-aminopteroylglutamic acid. That the folic acid offered some degree of protection against the toxic effects of the antagonist, however, was suggested by the following observations: (1) Treated animals tended to survive for a longer period, and (2) the bone marrow at death was cellular rather than hypoplastic, although nucleated erythrocytes

TABLE 4.—*Failure of Pteroylglutamic Acid and of Liver Extract to Prevent the Hematic Changes Caused in Guinea Pigs by Large Doses of 4-Aminopteroylglutamic Acid*

Guinea Pig	Daily Dose of 4-Amino-PGA Injected		Daily Dose of PGA,† Mg.	Liver Ext. UNP Units	Day of Exper.	Wt., Gm.	RBC $\times 10^6$ per Cu. Mm.		Hb., Gm.	WBC per Cu. Mm.		Lymphocytes per Cu. Mm.	Reticulocytes, %	Platelets $\times 10^6$ per Cu. Mm.	Survival, Days
	Total, Mg.	Mg. per Kg.*													
15	2.5	4.54	35	..	0	645	8.95	18.0	17,500	4,400	3.2	2,820			
					31	422	3.26	8.9	800	390	0	0	0	33	31
20	2.5	5.53	35	..	0	450	5.39	15.0	8,450	3,549	3.9	3,030			
					20	356	1.90	6.5	1,800	612	0	0	0	23	20
21	2.5	5.2	35	..	0	480	6.13	16.0	12,450	4,700	1.6	3,190			
	(for 34 days)				34	480	3.56	6.4	2,000	800	0	0	0	33	
					37	430	1.99	4.6	5,300	2,690	2.9	80			37
7	1.0	2.0	35	..	0	510	5.61	13.8	3,750	2,860	3.4	1,680			
			30‡		30	451	1.37	2.8	9,950	3,400	0.4	1,330			
			100‡		34	412	1.14	2.7	9,050	3,300	0	1,430			
					40	355	.81	3.3	11,300	900	0.2	944			41
13	0.5	0.97	35	..	0	515	6.01	12.5	7,100	4,600	5.8	2,388			
	(for 56 days)				56	486	1.07	3.3	19,500	4,800	0.6	417			59
9	1.0	2.2	..	35	0	450	5.02	14.4	9,900	6,150	3.3	1,350			
	(for 13 days)		30‡		13	375	1.38	3.3	10,850	5,200	0	1,070			
					18	302	.91	2.2	29,500	5,800	4.3	721			
					22	310	2.49	7.0	6,950	2,640	84.8	1,560			
					29	405	4.39	11.3	8,800	2,960	10.2	2,000			
					42	500	6.31	16.8	7,700	5,000	0.6	1,930			Survived
11	1.0	2.6	..	5	0	380	5.48	14.2	7,350	2,300	1.2	1,070			
	(for 19 days)				20	332	2.23	5.7	400	180	0	244			21
12	1.0	2.4	..	5	0	430	6.32	15.2	8,300	5,300	3.0	1,820			
	(for 23 days)				24	370	1.45	3.9	5,000	2,400	0	1,050			26

* This was calculated on the basis of the weight at the beginning of the experiment.

† Of the amount given, 5 mg. was injected; the remainder was given orally.

‡ The daily dose of pteroylglutamic acid was changed on the days indicated.

§ The liver extract was discontinued after 13 days.

remained frankly reduced in number. The predominant cell was a very primitive blast form. In dogs it was possible to inject 200 to 800 times more pteroylglutamic acid than 4-aminopteroylglutamic acid and to achieve a greater degree of protection.

Eight guinea pigs were given 4-aminopteroylglutamic acid in doses comparable to those used in the preceding experiments. Five of these animals received, in addition, amounts of folic acid that were 10 to 100 times larger than the dose of the antagonist; two were given a daily injection of 5 U. S. P. units of a purified liver extract,⁹ and one received both folic acid and liver extract. Only 5 mg. of each daily dose of folic acid was given subcutaneously; the remainder was administered by mouth. The results are recorded in table 4. In all instances the

9. Lederle Laboratories liver extract, 15 U. S. P. units per cubic centimeter.

loss of weight and the peripheral hematic changes were comparable to those observed in guinea pigs given 4-aminopteroylglutamic acid alone, and seven of the eight animals died. There was evidence, however, that the folic acid had afforded partial protection against the toxic effects of the antagonist. Guinea pigs 15, 20, 21, 7 and 13 survived for definitely longer periods than had animals given comparable amounts of 4-aminopteroylglutamic acid without folic acid supplementation. The bone marrow was also different. In guinea pigs 15, 20 and 7 it was normally cellular, but the differential counts showed roughly that one third of the cells were lymphocytes, one third myelocytes and one third "reticulum" cells. Megakaryocytes were found in approximately normal numbers in spite of the thrombocytopenia present in animals 15 and 20. Nucleated erythrocytes were rare; a few early erythroblasts were seen, but these did not possess the characteristics of the megaloblasts of pernicious anemia. The marrows of guinea pigs 13 and 21 differed in that they were hyperplastic and contained numerous normoblasts. This increase in normoblasts may well have occurred as the result of a beginning hematic recovery. Injections of 4-aminopteroylglutamic acid had been discontinued three days before death, and previous experiments (table 2, guinea pigs 14 and 18) had demonstrated that normoblasts return to the marrow promptly after administration of the antagonist is stopped.

Evidence of a protective effect of liver extract was not obtained. The survival times of animals given liver as well as 4-aminopteroylglutamic acid were not clearly prolonged. The bone marrow studies in animals 11 and 12 were made at the time of death, several days after the last injection of antagonist. The marrow was cellular in each instance; the percentage of normoblasts was higher than normal. It is not possible to tell how much of the cellularity resulted from the action of the liver extract and how much was due to release from the inhibitory effect of 4-aminopteroylglutamic acid.

One animal in this group recovered (guinea pig 9). Injections of the antagonist were discontinued on the thirteenth day, however, and it seems likely that the remission was spontaneous. It is possible that the liver extract and/or the pteroylglutamic acid might have influenced the height of the reticulocyte response, but the recovery did not differ otherwise from that observed in guinea pig 10 (table 2).

Hepatomegaly was found at necropsy in all guinea pigs given supplementary pteroylglutamic acid; autopsy findings otherwise were similar to those observed when 4-aminopteroylglutamic acid alone was administered.

Four separate experiments were done on three of the dogs that previously had received only the antagonist. These dogs offered two advantages: (1) because of the great sensitivity manifested by dogs to 4-aminopteroylglutamic acid, the doses could be kept rather small, and (2) the larger size of the animals made it possible to inject 200 mg. of folic acid per day. Consequently, a high ratio of pteroylglutamic acid to antagonist could be maintained: from 200:1 to 800:1. All animals survived. In only one instance, dog 4, were there a significant loss of weight, moderate dehydration, glossitis and diarrhea. Melena did not occur. Anemia, leukocytopenia and thrombocytopenia were not observed. In two experiments the lymphocytes decreased temporarily, and in three experiments there was a moderate depression of reticulocytes. From these results it would appear that extremely large doses of pteroylglutamic acid are able to offer a high degree of protection against the toxic effects of 4-aminopteroylglutamic acid. An objection may be made on the grounds that tolerance of 4-aminopteroylglutamic acid may

develop as a result of prior administration. Berman, Axelrod, VonderHeide and Sharpe,¹⁰ for instance, reported that patients with chronic leukemia could be treated with larger cumulative doses of the antagonist if small doses were used initially. Consistent with this observation is the fact that dog 4 (table 1), after an interval of 11 months, was able to tolerate 0.5 mg. 4-aminopteroylglutamic acid daily for six days much better than he had initially. However, the experiment in which dog 5 was given the antagonist by itself and in which toxic manifestations were severe (table 1) was done between the two pteroylglutamic acid supplemented experiments recorded in table 2.

COMMENT

The results of these experiments support the concept that 4-aminopteroylglutamic acid exerts its toxic effects by inducing an acute deficiency of folic acid. The possibility that its toxicity is mediated through some other mechanism is not ruled out, but seems less likely. When folic acid deficiency is induced in experimental animals fed a purified diet and given one of the sulfonamides to lessen synthesis on the part of intestinal organisms, the manifestations of deficiency are not identical in all species.¹¹ Loss of weight, anorexia, involvement of the gastrointestinal tract and varying degrees of interference with bone marrow function, however, are commonly observed. All these changes occur when 4-aminopteroylglutamic acid is given.

It is particularly interesting to compare the marrow changes observed in folic acid deficiency induced by nutritional means with those in the deficiency produced by administration of 4-aminopteroylglutamic acid. The marrow of the animals suffering from the former has been variously described as hypoplastic^{11f, 1} and as cellular. When it was cellular, some observers mentioned only an increase of nucleated red blood cells and/or an increase in the number of blast cells,^{11e, d} while others described an arrest of maturation of erythroid elements, some of which were regarded as having characteristics similar to the

10. Berman, L.; Axelrod, A. R.; VonderHeide, E. C., and Sharpe, E. A.: *J. Lab. & Clin. Med.* **33**:1643, 1948.

11. (a) Hogan, A. G., and Parrott, E. M.: *J. Biol. Chem.* **132**:507, 1940. (b) Franklin, A. L.; Stokstad, E. L. R., and Jukes, T. H.: *Proc. Soc. Exper. Biol. & Med.* **65**:368, 1947. (c) Weir, D. R.; Heinle, R. W., and Welch, A. D.: *Ibid.* **60**:211, 1948. (d) Franklin, A. L.; Stokstad, E. L. R.; Belt, M., and Jukes, T. H.: *J. Biol. Chem.* **160**:427, 1948. (e) Campbell, C. J.; Brown, R. A., and Emmett, A. D.: *Ibid.* **152**:483, 1944. (f) Endicott, K. M.; Daft, T. S., and Ott, M.: *Arch. Path.* **40**:364, 1945. (g) Spicer, S. S.; Daft, F. S.; Sebrell, W. H., and Ashburn, L. L.: *Pub. Health Rep.* **57**:1559, 1942. (h) Bethell, F. H.; Swendseid, M. E., and Rosenman, R. H.: *J. Clin. Investigation* **23**:926, 1944. (i) Gross, P.; Axelrod, A. E., and Bosse, M. D.: *Am. J. M. Sc.* **200**:642, 1944. (j) Heinle, R. W.; Welch, A. D., and Pritchard, J. A.: *J. Lab. & Clin. Med.* **33**:1647, 1948. (k) Cartwright, G. E.; Fay, J.; Tatting, B., and Wintrobe, M. M.: *Ibid.* **33**:397, 1948. (l) Doan, C. A.: *Am. J. M. Sc.* **212**:257, 1946.

megaloblasts of pernicious anemia.¹² When relatively large doses of 4-aminopteroylglutamic acid are given, as in the experiments described in this report, the marrow becomes hypoplastic. With amounts small enough to be tolerated over a period of weeks, however, the marrow tended to show increased cellularity with immaturity of both granulocytic and erythrocytic precursors.⁹ In addition, Thiersch and Philips¹³ reported that when serial bone marrow studies were done on dogs after the administration of 4-aminopteroylglutamic acid, they observed increasing degrees of hypocellularity, but before hypoplasia became advanced they were able to detect qualitative changes in nucleated red cells which made them resemble megaloblasts. A possible explanation of all these results is the following: With moderate degrees of folic acid deficiency the marrow may remain cellular and, in experimental animals, may even show evidences of maturation arrest. Hypoplasia of the marrow may be the result of advanced deficiencies. Similarly, with relatively small doses of 4-aminopteroylglutamic acid the folic acid deficiency produced may be mild and the marrow may remain cellular for that reason. Larger doses may precipitate an acute deficiency of more severe degree and lead rapidly, therefore, to marrow hypoplasia.

Protection against the effects of small amounts of 4-aminopteroylglutamic acid is apparently readily obtained by the simultaneous administration of folic acid.¹⁴ In rats partial protection is afforded against toxic doses of the antagonist by large amounts of folic acid.¹⁵ With daily administration of large amounts of folic acid we were not able to prevent the daily injection of 0.5 to 2.5 mg. of 4-aminopteroylglutamic acid from causing severe anemia in guinea pigs. In dogs, however, a high degree of protection was afforded when the amount of folic acid given was 200 to 800 times greater than the dose of antagonist.

One of the most interesting demonstrations provided by these experiments is the evidence for a significant difference in species susceptibility to the toxic effects of 4-aminopteroylglutamic acid. Dogs were much more sensitive than guinea pigs, and guinea pigs were more susceptible than rabbits. Cartwright and his associates,¹⁶ furthermore, have reported that pigs weighing 32 to 37 Kg. were able to tolerate

12. Heinle, R. W.; Welch, A. D.; George, W. L.; Epstein, M., and Pritchard, J. A.: *J. Lab. & Clin. Med.* **32**:1398, 1947. Cartwright and others.^{11k}

13. Thiersch, J. B., and Philips, F. S.: *Proc. Soc. Exper. Biol. & Med.* **71**: 484, 1949.

14. Swendseid, M. E.; Wittle, E. L.; Moersch, G. W.; Bird, O. D., and Brown, R. A.: *J. Biol. Chem.* **179**:1175, 1949.

15. Higgins, G. M.: *Blood* **4**:1142, 1949.

16. Cartwright, G. E.; Palmer, J. J.; Tatting, B.; Ashenbrucker, H., and Wintrobe, M. M.: *J. Lab. & Clin. Med.*, to be published.

16 mg. of 4-aminopteroylglutamic acid per day without any changes developing in the peripheral blood or the marrow. These differences become all the more striking when one recalls that the therapeutic dose of 4-aminopteroylglutamic acid for adult patients with acute leukemia is only 1 or 2 mg. per day, and that even this comparatively small amount may cause severe degrees of marrow hypoplasia.

Nichol and Welch¹ have recently presented evidence that 4-aminopteroylglutamic acid affects metabolism by interfering with the process by which folic acid is converted to the "citrovorum factor." This factor is the product of the metabolic alteration of pteroylglutamic acid and is a biologically active derivative of folic acid. It was shown to be capable of preventing the toxicity of 4-aminopteroylglutamic acid. The work

TABLE 5.—Effect of Simultaneous Administration of Pteroylglutamic Acid and 4-Aminopteroylglutamic Acid in Dogs

Dog	Daily Dose of 4-Amino-PGA Injected		Daily Dose of PGA,†	Day of Exper.	Wt., Kg.	RBC × 10 ⁶ per Cu. Mm.		Hb., Gm.	WBC per Cu. Mm.		Lymphocytes per Cu. Mm.		Etiocyto- cytes, %		Platelets × 10 ⁶ per Cu. Mm.		Survival, Days
	Total, Mg.	Mg. per Kg.*															
6	0.5	.044	200	0	11.4	6.60	15.8	18,150	1,603	2.0	1,800	Survived
	(for 5 days)			5	11.4	6.90	17.2	14,300	2,414	4.2	
	1.0	.088	200	10	10.7	7.50	19.0	22,800	229	2.4	1,717	
	(for 5 days)			17	9.0	8.08	19.6	17,750	1,850	0.4	2,245	
				41	12.5	7.30	17.3	15,200	3,657	0.6	1,930	
5	0.5	.046	200	0	11.1	7.02	17.6	11,000	800	2.5	1,744	Survived
	(for 12 days)			12	9.8	7.27	17.5	20,000	800	0.4	2,230	
				29	11.0	6.95	15.2	20,350	1,628	2.6	2,400	
	1.0	.097	200	0	10.3	7.35	17.6	18,000	2,100	1.4	2,237	
	(for 7 days)			7	10.4	6.81	17.3	12,600	1,300	2.4	2,010	
7				23	10.9	6.76	17.8	12,900	1,806	0.6	1,800	Survived
	0.25	.020	200	0	12.3	7.10	17.3	3,620	1,917	0.6	502	
	(for 12 days)			14	11.5	6.60	17.0	14,500	2,175	1.8	1,100	
				28	11.0	6.29	16.6	14,000	2,500	1.4	1,460	

* This was calculated on the basis of the weight at the beginning of the experiment.

† The pteroylglutamic acid was injected subcutaneously.

of these two authors suggests in addition that 4-aminopteroylglutamic acid interferes with the utilization of the citrovorum factor. It is not clear at present, on the basis of the above explanation, why different species of animals should manifest such a striking difference of susceptibility to 4-aminopteroylglutamic acid.

Lymphocytopenia was observed in the majority of animals studied in this investigation. Dougherty and Dougherty¹⁷ have suggested that 4-aminopteroylglutamic acid has a hormonally mediated effect as well as a direct inhibitory action on the lymphatic system because they found that lymphocytopenia and atrophy of lymphatic tissue did not occur in mice adrenalectomized prior to administration of antagonists. Other authors, however, have not been willing to accept the idea that 4-amino-

17. Dougherty, J. H., and Dougherty, T. H.: J. Lab. & Clin. Med. **35**:271, 1950.

pteroylglutamic acid stimulates pituitary and adrenal cortical secretions.¹⁸ The observations reported in the present paper are not helpful in resolving this disagreement.

SUMMARY AND CONCLUSIONS

A distinct difference in the susceptibility of dogs, guinea pigs and rabbits to the toxic effects of 4-aminopteroylglutamic acid was demonstrated. Dogs were the most sensitive of the three species of animals. The acute changes produced by relatively large doses of the antagonist are described. With large amounts, hypoplasia of the marrow was an invariable result. The reasons for regarding the manifestations of 4-aminopteroylglutamic acid toxicity as evidences of an acute folic acid deficiency are discussed, and a possible mechanism of action of 4-aminopteroylglutamic acid is reviewed. Large amounts of folic acid were able to provide a high degree of protection in dogs against doses of the antagonist which otherwise were lethal. The protection achieved in guinea pigs was less complete.

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THE MOVEMENT OF WATER IN INTERSTITIAL TISSUE AND IN MUSCLE REMOVED FROM THE BODY

EUGENE L. OPIE, M.D.

AND

MARY BOON ROTHBARD, M.D.

NEW YORK

THE PRESENT study has been undertaken in order to obtain information about some of the characteristics of interstitial tissue that are concerned with the movement of water during life. In previous publications¹ the water exchange of tissues removed from the body and immersed in various fluids has been described, and conspicuous differences in this relation between parenchymatous tissues, namely liver, pancreas and kidney, on the one hand, and loose interstitial tissue of omentum or thymus, on the other hand, have been defined. It is noteworthy that organs chiefly composed of secreting cells are isotonic with solutions of sodium or potassium chloride of approximately twice the concentration of sodium chloride in the blood, whereas loose areolar tissues (omentum, thymus) are isotonic with blood serum. The hepatomas and sarcomas that have been examined are isotonic with solutions of sodium chloride of approximately the same concentration as that in blood. Unlike the areolar tissues mentioned, dense fibrous tissue of the corium and of the aorta and the tough, fibrous stroma of benign fibroadenomas when immersed in solutions of sodium chloride fail to reach water equilibrium with increasing concentration and take up water in all solutions from 0.1 to 0.6 molar.

METHODS

Particles of tissue have been weighed on a torsion balance and, after immersion in various fluids, reweighed at intervals in order to determine directly how much water has entered or left them. The details of the method have been described elsewhere.¹ The rats from which tissues have been obtained have received a diet consisting of bread, milk and carrots and have had access to water. They have been killed by bleeding, and blood has been removed from the tissue

From the Laboratories of The Rockefeller Institute for Medical Research.

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1. Opie, E. L.: *J. Exper. Med.* **89**:185 and 209, 1949.

slices by bringing them into contact with cotton gauze. Slices of muscle are not so readily cut as those of liver or kidney and are often irregular in shape. They have usually been prepared from rectus or psoas muscle and have weighed from 75 to 150 mg. In some instances small muscles have been isolated by dissection, and the whole muscle has been used for immersion. The muscular part of the diaphragm, cut into five or six pieces, has been used. Corium has been obtained from the thick skin of the back after removing the hair with clippers and dry shaving. The closely attached cutaneous muscle has been completely removed by dissection with a sharp knife. The corium, still covered on one side by epithelial cells, is cut into rectangular pieces approximately 75 by 150 mm. and weighing from 100 to 125 mg. The percentage change of weight is a measure of the movement of water. The tissue is soon profoundly modified by the injury following immersion, and in most of the experiments observations have been limited to the initial water exchange, occurring with the first 10 minutes.

MOVEMENT OF WATER IN CORIUM OF SKIN REMOVED FROM THE BODY

The intercellular material of connective tissue consists of collagenous and elastic fibrils, cement substance collecting the fibrils into bundles, amorphous ground substance, perhaps continuous with the cement substance, and tissue fluid, often assumed to occupy definable tissue spaces. The homogeneous semigelatinous ground substance which in the embryo precedes the formation of connective tissue fibers is well known, and there is a transition in physical characters from the soft, gelatinous material represented by "Wharton's jelly" of the umbilical cord through areolar and dense fibrous tissue to cartilage and bone. Observations of the transparent tail fin of amphibian larvae and of connective tissue developing within transparent chambers in the rabbit's ear by Clark and Clark² have furnished evidence that the intercellular substance is gelatinous and not fluid. India ink particles and fat droplets remain sharply localized when introduced by a micropipet, whereas within the lumen of a lymphatic or blood capillary they spread instantly. A significant observation has been made by McMaster.³ Locke's solution or blood serum was introduced with a fine needle into the cutaneous connective tissue of mice. At low pressures a small quantity of fluid entered the tissue, but the rate of flow was not increased until a pressure of 8.5 cm. of water was reached. At this level, designated the "breaking point," the flow was abruptly accelerated and later increased with relation to further elevation of pressure.

Water penetration of connective tissue immersed in various solutions was studied by Schade and Menschel,⁴ who found that interstitial tissue of the umbilical cord and tendon, under some conditions, became swollen even in strong solutions of sodium chloride, and they measured roughly the pressure necessary to prevent swelling. Their observations were

2. Clark, E. R., and Clark, L. C.: *Am. J. Anat.* **52**:273, 1933.

3. McMaster, P. D.: *J. Exper. Med.* **74**:9, 1941.

4. Schade, H., and Menschel, H.: *Ztschr. f. klin. Med.* **96**:279, 1923.

made by weighing tissues at intervals of 24 hours and gave little information about the initial changes that occurred before the tissues were subjected to the injury associated with prolonged immersion in various fluids.

When corium of the skin is immersed in solutions of sodium or of potassium chloride, it takes up water from concentrations varying from 0.1 to 0.6 molar or more (chart 1). Solutions of sucrose, a nonelectrolyte, cause initial movement of water which varies with the concentration of the solution and when plotted follows an approximately linear course (chart 1). A solution of sucrose approximately 0.36 molar has been in water equilibrium with the tissue, and water has been drawn from it by stronger solutions. Water exchange of corium in solutions of urea, which has small molecular weight, is highly diffusible and is in

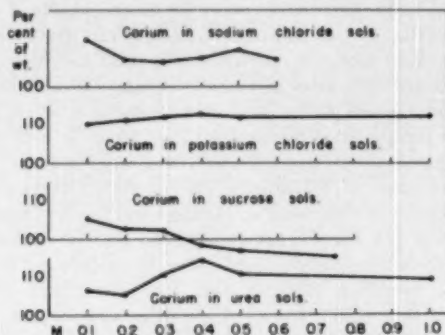


Chart 1.—Initial changes in per cent of weight of corium of the skin when immersed (a) in solutions of sodium chloride varying in concentration from 0.1 to 0.6 molar; (b) in solutions of potassium chloride, from 0.1 to 1.0 molar; (c) in solutions of sucrose, from 0.1 to 0.75 molar, and (d) in solutions of urea, from 0.1 to 1.0 molar. The period of immersion was 10 minutes.

some degree subject to electrolytic dissociation, resembles that in solutions of sodium and of potassium chloride (chart 1).

MOVEMENT OF WATER IN TENDINOUS FASCIA REMOVED FROM THE BODY

The powerful thoracic and lumbar muscles of the back of the rat are covered by a conspicuous widespread tendinous fascia by which they are attached to the bones of the vertebral column. With a sharp knife, this fascia can be separated from the underlying muscle. It has been divided into pieces of suitable size and weighed before and after immersion in solutions of sodium chloride. Like corium, it has taken up water in solutions varying from 0.1 to 0.75 molar (chart 2), and the proportional quantity of water taken in has been considerably greater than that with

corium. Intake of water after immersion of this fascia in sacrose solutions is much less than in sodium chloride and, though it diminishes with increasing concentration of the sacrose solution, does not reach isotonicity within the range of concentrations used.

The changes which occur when striated muscle is immersed in distilled water differ conspicuously from those of other tissues under the same conditions and will be described. They are dependent on certain physical properties of excised muscle but obviously have little resemblance to water exchange within the living body.

Swelling of muscle in water has been described by Fischer⁵ and Meigs.⁶ The tissue takes up water very rapidly during the first half-hour of immersion and then loses water with almost equal rapidity;

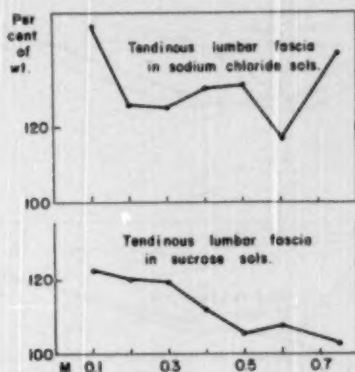


Chart 2.—Initial changes in the per cent of weight of tendinous fascia (a) in solutions of sodium chloride and (b) in sacrose solutions varying in concentration from 0.1 to 0.75 molar. The period of immersion was 10 minutes.

later the weight increases. When frog's muscle was immersed in solutions of sodium chloride, Adolph⁷ found that in an initial period the intake of water was proportional to the square root of the time of immersion, and Parry⁸ observed the same relation when he studied mammalian muscle. Buglia⁹ immersed striated muscle of the frog in solutions of sodium chloride and found that it was isotonic with a solution 0.125 molar.

5. Fischer, M. H.: *Arch. f. d. ges. Physiol.* **124**:69, 1908.

6. Meigs, E. B.: *Am. J. Physiol.* **26**:191, 1910.

7. Adolph, E. F.: *Am. J. Physiol.* **96**:569, 1931.

8. Parry, A. A.: *J. Cell. & Comp. Physiol.* **8**:277, 1936.

9. Buglia, G.: *Arch. internat. physiol.* **8**:273, 1909.

WATER PENETRATION INTO STRIATED MUSCLE IMMERSSED IN DISTILLED WATER, RINGER'S SOLUTION OR BLOOD SERUM

The changes which occur when striated muscle is immersed in water differ from those of other tissues under similar conditions. The latter's intake of water causes gradual increase of weight, and a maximum is reached after one or two hours. Following immersion of slices of striated muscle from the rectus or the psoas muscle (chart 3), there is rapid increase of weight which may reach a maximum five minutes after

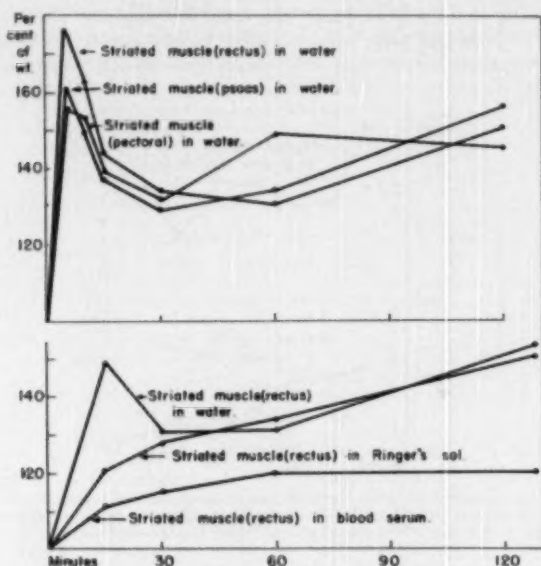


Chart 3.—Upper graph shows changes in the per cent of weight of slices of striated muscle of rectus, psoas and pectoral muscles in water during two hours. Lower graph shows changes in the per cent of weight of slices of rectus muscle in water, in Ringer's solution and in blood serum of rat.

immersion. This peak increase of weight is followed by a precipitate fall but later, after one half to one hour, the weight increases slowly.

Muscle takes up water in isotonic sodium chloride solution (approximately 0.15 molar), Ringer's solution (charts 3 and 4), which represents the molecular concentration of the electrolytes in blood plasma, and in blood serum of the rat (chart 3). In these solutions there is no peak increase of weight within the first few minutes after immersion as in water but a gradual increase which reaches a maximum after one or two

hours. In blood serum the muscle takes in less water than in Ringer's solution; the difference is presumably referable to the protein content of the serum.

MOVEMENT OF WATER IN STRIATED MUSCLE REMOVED FROM THE BODY

When striated muscle, as for example rectus, psoas or pectoralis major, is immersed in solutions of sodium chloride of increasing concentration (chart 5) water exchange is not approximately proportional, as with liver and kidney,¹ to the concentration of the solution, with isotonicity at one level and loss of water in stronger solutions, but resembles that of corium or aorta, with intake of water even in very strong solutions (0.6 molar). In some, but not in all, instances (see charts 5 and 6) water intake diminishes as the concentration increases

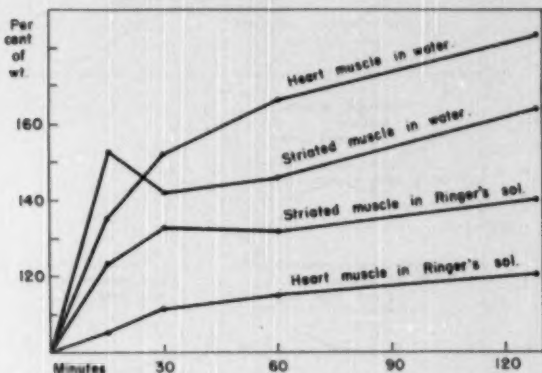


Chart 4.—Changes in the per cent of weight of slices (a) of striated muscle in water and in Ringer's solution and (b) of heart muscle in water and in Ringer's solution.

to 0.3 or 0.4 molar, and then reaches a higher level with stronger solutions. Water intake of striated muscle in solutions of potassium chloride (chart 6) closely resembles that in sodium chloride, being in both dependent on molecular concentration.

Water exchange of striated muscle in solutions of sucrose is like that of corium and when plotted (chart 6) pursues a linear course in relation to concentration. Muscle has drawn water from the strongest solutions of sodium chloride that have been used, but with increased concentration of sucrose water intake diminishes and equilibrium occurs at some level between 0.4 and 0.5 molar.

In a few instances water intake of striated muscle has followed the usual course of osmosis and has decreased with increasing concentration

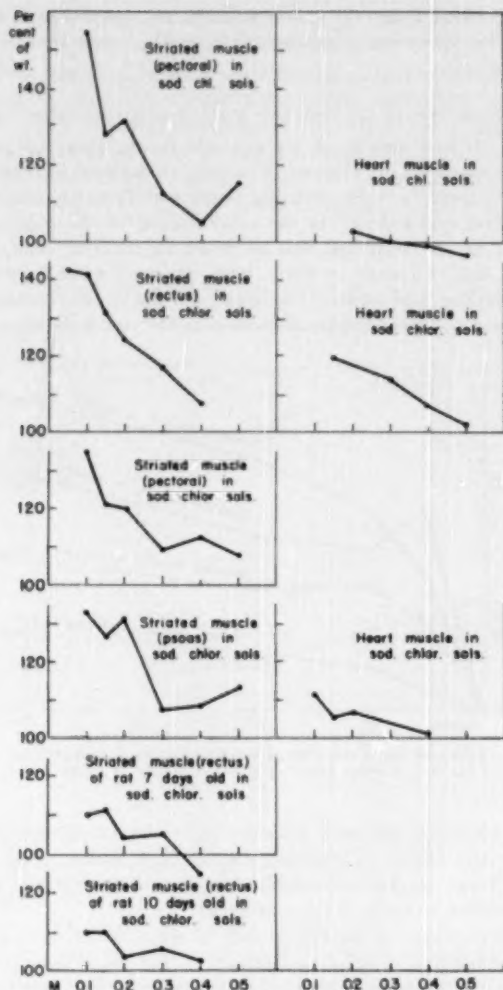


Chart 5.—(a) Four graphs show initial changes in the per cent of weight of slices of adult striated muscle in solutions of sodium chloride, varying from 0.1 to 0.6 molar. The period of immersion was 15 minutes. (b) Two graphs show initial changes in the per cent of weight of slices of striated muscle from two very young rats, the period of immersion being 10 minutes. (c) Three graphs show initial changes of weight of slices of heart muscle after 10 minutes of immersions.

until equilibrium has been reached or water withdrawn. Some whole small muscles suitable for immersion and weighing have lost water in solutions of relatively high concentration and have evidently been isotonic with less concentrated solutions. A part of the pectoralis minor muscle, designated by Greene¹⁰ as the third portion, is readily isolated and is free from tendinous strands, whereas the tibialis posterior, also readily isolated, is tough and conspicuously tendinous. These muscles from four animals have been carefully isolated and immersed in solutions of

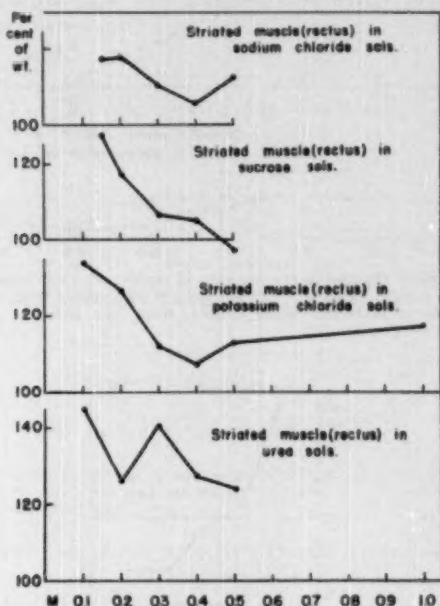


Chart 6.—The initial change of weight of striated muscle (a) in solutions of sodium chloride varying in concentration from 0.1 to 0.5 molar; (b) in solutions of sucrose; (c) in solutions of potassium chloride, 0.1 to 1.0 molar, and (d) in solutions of urea.

sodium chloride (chart 7). The tibialis posterior has taken up water from all solutions, varying in concentration from 0.15 to 0.6 molar, but the pectoralis minor in two instances lost water in solutions 0.5 and 0.6 molar and presumably tends to reach equilibrium in solutions of somewhat lower concentration. Diaphragm (chart 8) cut into small pieces,

10. Greene, E. C.: *Anatomy of the Rat*, in *Transactions of the American Philosophical Society*, Philadelphia, University of Pennsylvania Press, 1935, vol. 27.

weighing approximately 100 mg., has lost water in the higher concentrations of sodium chloride that have been used and is isotonic with solutions between 0.2 and 0.3 molar; whereas in sucrose it has similarly lost water in strong solutions and has been isotonic with solutions between 0.3 and 0.4 molar.

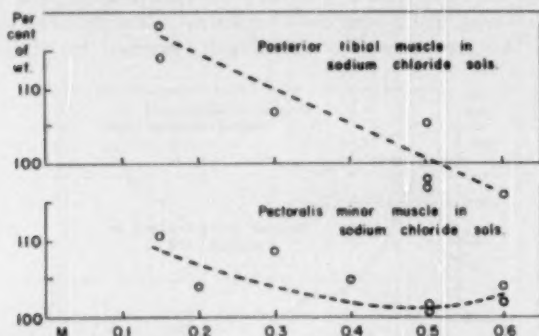


Chart 7.—Initial changes in the per cent of weight of whole muscle immersed in solutions of sodium chloride: (a) posterior tibial muscles from four animals; (b) third parts of pectoralis minor muscles from four animals. The time of immersion was 10 minutes.

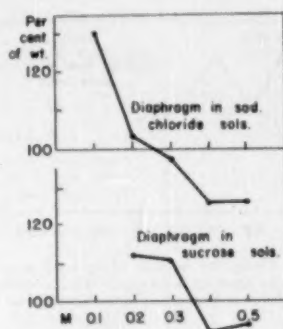


Chart 8.—Initial changes in the per cent of weight of segments of the muscular part of the diaphragm (a) in solutions of sodium chloride and (b) in solutions of sucrose. The time of immersion was 10 minutes.

In two instances slices of rectus muscles of very young rats, 7 and 10 days old, have been immersed in solutions of sodium chloride (chart 5). Water intake of one of them has diminished with increasing concentration until equilibrium has been established at a level representing 0.36 molar sodium chloride.

MOVEMENT OF WATER IN HEART MUSCLE REMOVED FROM THE BODY

Heart muscle immersed in water (chart 4) has not taken it up with the rapidity observed when striated muscle has been similarly placed, but weight of immersed slices has gradually increased and reached a maximum after two to three hours. In Ringer's solution (chart 4) heart muscle has taken up water, but the proportional increase of weight has been much less than with striated muscle. In solutions of sodium chloride water intake of heart muscle (chart 5) has decreased with increasing concentration and, unlike striated muscle, tends to reach isotonicity in concentrations greater than 0.3 molar.

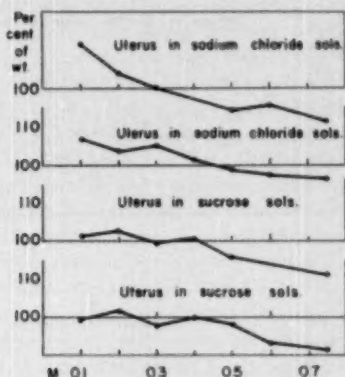


Chart 9.—Initial changes in per cent of weight of tissue of uterus. Tissue from two animals (a) in solutions of sodium chloride and (b) in solutions of sucrose. The time of immersion was 10 minutes.

MOVEMENT OF WATER IN TISSUE OF THE UTERUS REMOVED FROM THE BODY

Uterus has been prepared for immersion in fluids preferably by opening the lumens of the two horns, drying the mucous surface with cotton gauze and dividing into pieces of appropriate size. Chart 9 shows the results of its immersion in solutions of sodium chloride and of sucrose. In both solutions intake of water is scant and when plotted follows an approximately linear course, with isotonicity varying considerably. The uteri of two animals weighing more than 200 Gm. have taken up water when immersed in sodium chloride solutions in greater proportional quantity, and water intake has not diminished in high concentrations.

MOVEMENT OF WATER IN NERVE TRUNKS REMOVED FROM THE BODY

Water intake of large nerve trunks, namely, anterior crural and sciatic nerves, has been compared with that of muscle because these nerves consist of nerve fibrils within a framework of connective tissue. The pieces used have been small, and the results are only approximately correct. In sodium chloride (chart 10) water intake has been conspicuous in all concentrations up to 0.75 molar, whereas in sucrose it has decreased with concentrations greater than 0.4 molar. The water

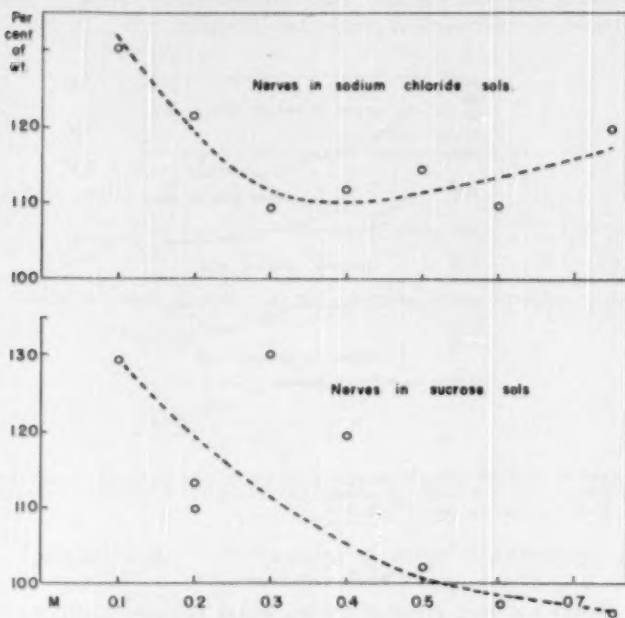


Chart 10.—Initial changes in the per cent of weight of pieces of nerve trunks immersed (a) in solutions of sodium chloride and (b) in solutions of sucrose. The time of immersion was 10 minutes.

exchange has resembled that of muscle, but its relation to nerve fibers and connective tissue framework is not evident.

RECAPITULATION AND COMMENT

The water exchange which immediately follows the immersion of liver or kidney tissue in solutions of sodium chloride, used as a measure of osmosis, varies with the concentration of the solution and is like that which occurs when water passes through a semipermeable membrane.

The molecular concentration within the cells of these organs is indicated by their isotonicity with solutions of sodium chloride and is approximately twice that of a solution isotonic with blood plasma. The water exchange of dense fibrous tissue, such as that of the corium of the skin, is not proportional to the concentration of the solutions of sodium chloride in which it is immersed, and the tissue is like gelatin, which under certain conditions, as Kunitz¹¹ found, swells as the result of water intake in both weak and strong solutions of the electrolyte.

The attempt was made to obtain more information about the water exchange of fibrous tissue and, later, of muscle tissue. It soon became evident that movement of water within striated muscle immersed in solutions of sodium or of potassium chloride did not, as with liver and kidney, resemble osmotic interchange through a semipermeable membrane but, in most instances, was like that of the corium or of the aortic wall. The water intake of the tendinous fascia of striated muscle was found to be the same as that of other dense fibrous tissues. Corium of the skin takes up water from solutions of sodium or of potassium chloride, varying from 0.1 to 0.75 molar. When it is immersed in solutions of a nonelectrolyte of large molecular size, namely, sucrose, its intake of water is much less and diminishes with increasing concentration. Water intake of tendinous fascia in solutions of sucrose is similarly reduced. Solutions of urea, on the contrary, have increased water intake above that with sodium chloride.

The water intake of powerful muscles, including rectus, psoas and pectoralis major muscles, in sodium chloride solutions closely resembles that of corium or of isolated tendinous fascia, but there is often diminution of swelling in solutions from 0.1 to 0.3 or 0.4 molar and there is increase in stronger solutions. Nevertheless, in some instances the water intake more closely resembles the usual course of osmosis and has diminished continuously with increasing concentration of solutions. The water exchange of two small muscles that can be isolated and weighed has been compared; the tibialis posterior, penetrated by conspicuous tendinous strands, has taken up water from stronger solutions, but the third part of the pectoralis minor, consisting of soft muscle bundles, has lost water in strong solutions. The intake of segments of diaphragm when plotted is in approximately linear relation to the concentration of sodium chloride, with isotonicity between 0.3 and 0.4 molar. Water exchange of the rectus muscle of a rat 7 days old, unlike that of adult animals, has proceeded in accord with the usual course of osmosis.

Movement of water within adult striated muscle is evidently dependent on two factors: (1) osmotic interchange of muscle cells and (2) hydration with swelling of interstitial tissue, represented in the more

11. Kunitz, M.: *J. Gen. Physiol.* **12**:289, 1928.

powerful muscles by tendinous fascia. In solutions of sucrose, osmotic interchange occurs, but hydration of interstitial tissue is much diminished.

A peculiar character of striated muscle is revealed under conditions that have little resemblance to any present during life, that is, immersion in distilled water. Liver, kidney, pancreas and adrenal gland in water¹ gradually increase in weight, reach a maximum after one or two hours and then lose weight. Heart muscle follows the same course. In contrast with these changes, adult striated muscle of the rectus, psoas or pectoralis major type, increases in weight with great rapidity, reaches a peak within five minutes, falls precipitately and later, after one-half to one hour, slowly increases in weight. In Ringer's solution or in blood serum no rapid swelling occurs, but weight increases gradually. No explanation of this sudden intake of water is evident.

CONCLUSIONS

When dense fibrous tissue like that of corium of the skin, wall of the aorta or tendinous fascia of muscle is immersed in solutions of sodium chloride, it swells by hydration; it takes up water in both weak and strong solutions of sodium chloride, in Ringer's solution and in blood serum.

In striated muscle under the same conditions movement of water occurs (1) by hydration of the fascial framework and (2) by osmotic interchange between muscle fibers and the surrounding fluid.

Interchange of water in heart muscle and in smooth muscle of the uterus similarly immersed is, under usual conditions, chiefly by osmosis.

The water of interstitial tissue is normally in such relation to colloids that it is not freely movable in tissue spaces.

CANCER AND AGING

A Survey of the Autopsy Records of a Municipal Hospital Over a Fifteen Year Period

JOHN A. SAXTON Jr., M.D.

FRED P. HANDLER, M.D.

AND

JOHN BAUER, M.D.

ST. LOUIS

THE DIFFICULTIES and errors inherent in the statistical study of human tumors have been thoroughly discussed by Willis.¹ They have as their main roots the degree of accuracy of diagnosis and the degree to which the sample is adequate both as to numbers and representation. According to Willis, the accuracy of diagnosis may be graded on the basis of the methods used in making the diagnoses, the greatest accuracy being reached by autopsy with histological confirmation. Ideally, as may obtain in longevity experiments with animals, post-mortem examination of all individuals should be made. Practically, it has not been possible except in extremely rare instances to approach this degree of representation in a human community. However, patients in a large municipal hospital will be subject to a lesser degree of selection than those in a private or a specialized hospital, and those who die there are thus most nearly representative of the mortality of the community. The degree of representation will then depend on the percentage coming to autopsy.

We have made a survey of the autopsy records of 12,443 white persons coming to autopsy in the municipal and state hospitals in St. Louis² during the past 15 years with the purpose of obtaining information on the following questions.

This work was aided by an Institutional Grant from the American Cancer Society to the Washington University School of Medicine.

From the Snodgrass Laboratory of the St. Louis City Hospital Division and the Department of Pathology, Washington University School of Medicine.

1. Willis, R. A.: Pathology of Tumors, St. Louis, C. V. Mosby Company, 1948.

2. St. Louis City Hospital, general municipal, white, 1,125 beds; St. Louis State Hospital, mental diseases, 3,400 beds; City Infirmary Hospital, chronic diseases, 1,475 beds; St. Louis; Robert Koch Hospital, tuberculosis, 650 beds, Koch, Mo.; St. Louis State Training School, mental defectives, 400 beds.

1. What is the autopsy incidence of cancers in a municipal hospital?
Is it changing in recent years?
2. What are the most common types of cancers found at autopsy?
Is the incidence of certain types of cancers changing in recent years?
3. Does the total frequency of cancers found at autopsy increase
directly and indefinitely as a function of age?
4. Do certain types of cancers found at autopsy have a characteristic
age incidence?
5. What is the frequency of multiple primary tumors in relation to
age?

TABLE 1.—Age and Sex Distribution of White Persons Coming to Autopsy Between July 4, 1935 and July 4, 1950 Whose Cases Were Surveyed in This Study, with the Incidence of Cancers in Each Age Group

Age Group, Yr.	Persons in Group		Males in Group		Females in Group		Persons with Cancer in Group		Males with Cancer in Group		Females with Cancer in Group	
	No.	%*	No.	%†	No.	%‡	No.	%§	No.	%	No.	%¶
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
0-9.....	1,231	9.8	679	8.7	542	11.6	8	0.66	4	0.6	4	0.7
10-19.....	187	1.5	100	1.2	87	1.8	11	5.9	6	6.0	5	5.8
20-29.....	377	3.2	161	2.1	216	4.6	18	4.8	11	7.0	7	3.2
30-39.....	667	5.3	379	4.9	288	6.1	71	10.6	39	8.0	41	14.2
40-49.....	1,300	9.6	720	9.3	480	10.3	197	16.4	96	12.5	107	22.3
50-59.....	3,063	16.2	1,586	17.9	647	14.8	439	21.6	390	23.6	139	21.5
60-69.....	2,661	23.5	1,964	25.5	967	21.0	772	28.2	525	26.7	247	25.0
70-79.....	2,677	21.4	1,739	22.4	938	20.9	586	21.8	397	22.8	189	20.2
80-89.....	1,948	9.4	989	7.6	459	9.8	303	19.3	117	19.9	86	18.7
90-99.....	82	0.6	42	0.54	40	0.8	17	20.7	11	26.2	6	15.0
Total.....	12,443	7,759	62.3	4,684	37.7	2,322	18.7	1,491	19.5	831	17.8

* Of all persons coming to autopsy (see foot of column 2).

† Of all males coming to autopsy (see foot of column 4).

‡ Of all females coming to autopsy (see foot of column 6).

§ Of all persons in the age group (see column 2).

|| Of all males in the age group (see column 4).

¶ Of all females in the age group (see column 6).

COMPOSITION OF THE GROUP

A total of 12,443 white patients, ranging in age from prematurity to a stated 105 years, were examined post mortem from July 4, 1935 to July 4, 1950. As shown in table 1, 2,322 of these bore cancerous tumors. In 85 patients there were two or more cancers, three being present in two persons. Thus a total of 2,409 cancers were encountered. The primary site could not be determined with certainty in 70 patients, or 3 per cent of those with cancers. None of these was classified as multiple, and in most of the persons examined the regional site was evident, although the organ or tissue of origin might be obscure.

These 12,443 patients constituted nearly one half of those who died, excluding those whose deaths were accidental or felonious and those

who died within less than 24 hours after receiving medical attention. The latter groups came under the jurisdiction of the city coroner, and approximately 30 per cent of deaths in the general municipal hospital were necessarily referred to him. Of the cases without medicolegal interest, on which this survey is based, permission to make an autopsy was solicited in all, irrespective of clinical interest; and the autopsy percentage at the general hospital has been kept at about 50 per cent in each of the last 15 years. If greater effort was made to secure permission in cases presenting a confusing clinical picture, this involuntary selection was not biased in favor of cases of cancer generally, or of any type of cancer in particular.

As a control for the adequacy of the sample, the age distribution of all white decedents of this community in the last 15 years, compiled by the Vital Statistics Bureau of the St. Louis Division of Health, was compared with that of the cases here surveyed. The comparison is shown in chart 1, and it is apparent that the age distribution of the cases surveyed (12,443) closely paralleled that of the total reported mortality of the community (135,724). Slightly more than 9 per cent of white persons dying in the community were thus included in the survey.

In two respects the cases surveyed might appear to have been weighted in favor of cancers. The exclusion of cases subject to legal inquiry because of short illness probably removed a greater number of persons who died suddenly of cerebrovascular or cardiac disease than of persons who died with cancer, because the latter tends to run a more chronic course. Because indigence is unfortunately common in the old, there is a preponderance of elderly persons in municipal hospitals or municipal custody, and cancer is an important disease of old age. However, it is probable that such factors of selection would act evenly over the period of time covered by the survey.

In order to list comparative data, the series was divided into three five year periods wherever practicable. Patients of the Negro race, 684, were omitted in order to include them in a comparable report at a later time.

DIAGNOSTIC CRITERIA

In all cases of this survey, microscopic sections were prepared and studied. Types of disease regarded as cancerous included not only the accepted varieties of carcinomas and sarcomas but also Hodgkin's disease, leukemias and brain tumors. Salivary gland tumors were recorded only if invasion or metastasis had occurred. Tumors found incidental to autopsy, if satisfying the criteria of cancer, were included, although they might not have contributed to the death of the patient. On the other hand, lesions of equivocal nature, such as preinvasive

carcinoma of the cervix uteri, simple papilloma of the urinary bladder and small carcinoma-like foci of the prostate without demonstrable invasion, were excluded. If the latter group had been added, there would have been many more cases of carcinoma of the prostate, and the number of patients with multiple cancers would have been greatly increased. Those patients who died free of discoverable tumor, but who had been treated surgically for a tumor proved to be a cancer (other than of the skin) were included in the survey. Microscopic sections of the surgical specimen were reviewed in each case. It may be said without

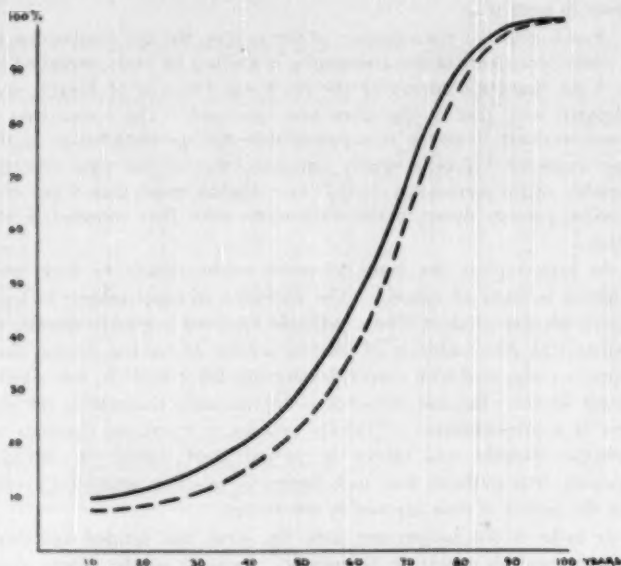


Chart 1.—Age distribution of 12,443 autopsies in the municipal and state hospitals of St. Louis (solid line) compared with age distribution of 135,724 white persons dying in the community (interrupted line) over the same period of 15 years.

undue pessimism that the inclusion of such cases did not materially alter the statistics of tumor incidence. The tabulations are least reliable where tumors of the skin are concerned, as their curability rate is high and the history frequently inadequate.

METHODS OF ANALYSIS

The necessary data of each autopsy were placed on individual punch cards, and these were sorted mechanically by the machine records

department of Washington University. The distribution of the cases in each five year period according to age (in decades) and sex, and the number of cancers of each type or organ of origin, including multiple cancers, with their distribution according to age and sex of the host, were thus determined. These assembled data formed the basis of the tabular and graphic analyses. The accuracy of the mechanical sorting was indicated by the almost complete absence of record of inconsistencies.

RESULTS

1. The autopsy incidence of cancers in a municipal hospital over a 15 year period.

In table 1 are shown the distribution of the patients coming to autopsy according to age and sex, and the incidence of cancers in each age group according to sex. It will be noted that nearly one fifth (18.7 per cent) of all patients coming to autopsy in the present series, regardless of age, bore cancers, and that the total frequency was slightly higher in men than in women.

The frequency of cancers in each of the five year periods surveyed is shown in table 2. There was a consistent increase in total frequency from 16.8 per cent in 1935-1940 to 20.6 per cent in 1945-1950. In table 2 it can also be seen that there was a progressive shift in distribution of patients in the direction of the older age group. That the increase in frequency was not purely dependent on the increasing age of the group surveyed is indicated by the almost uniform increase in tumor incidence within each age group of both sexes. It is, moreover, improbable that progressive accuracy of postmortem examination could have accounted for all of the increase.

2. The autopsy incidence of the most common types of cancers in a municipal hospital over a 15 year period.

In table 3 is a list of the 25 most common primary cancers or sites of origin arranged according to frequency in the total series of autopsies, with comparative figures for each five year period. The incidence of the 10 most common sites of cancers is calculated in per cent of the corresponding number of patients examined, irrespective of age or sex, and the order of frequency is indicated numerically for each five year period. Relative increases in frequency are indicated by decreased values of the ordinal numbers in successive five year periods, and, conversely, decreases are shown by increased values of the ordinals. The cancers, or sites of origin, which occurred in a frequency too low to merit tabulation included myeloma (17 cases), Hodgkin's disease (15 cases), adrenal gland (14 cases), lip (11 cases), pleura or peritoneum (10 cases), small intestine (9 cases), thyroid gland (9 cases), nasal passages and sinuses (9 cases), melanoma (8 cases), female external genitalia (8 cases) and penis (8

cases). There were five or less examples of cancers of the following sites: anus, carotid body, lacrimal gland, pituitary gland, salivary gland, skeletal system, testis, trachea, ureter, urethra and vagina.

TABLE 2A (First Five Year Period).—Age and Sex Distribution of White Persons Coming to Autopsy Between July 4, 1935 and July 4, 1940 Whose Cases Were Surveyed in This Study, With the Incidence of Cancers in Each Age Group *

Age Group, Yr.	Persons in Group		Males in Group		Females in Group		Persons with Cancer in Group		Males with Cancer in Group		Females with Cancer in Group	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
0-9.....	491	10.5	275	9.3	216	12.9	5	1.0	3	1.1	2	0.9
10-19.....	99	2.1	53	1.7	45	2.7	6	6.1	3	5.7	3	6.6
20-29.....	196	4.2	81	2.7	115	6.8	10	5.1	6	7.4	4	3.5
30-39.....	234	7.1	300	6.5	134	8.0	23	7.0	11	5.5	12	8.9
40-49.....	555	11.9	329	11.0	226	13.5	91	14.8	32	9.7	59	21.7
50-59.....	676	18.7	618	20.6	258	15.4	177	26.2	125	20.2	52	20.1
60-69.....	1,062	22.8	733	24.5	330	19.7	304	28.8	198	25.6	76	23.0
70-79.....	790	16.9	544	18.2	246	14.7	173	21.9	139	25.9	43	17.5
80-89.....	245	5.3	144	4.8	101	6.0	41	16.7	36	18.0	15	14.9
90-99.....	15	0.3	8	0.3	7	0.4	3	20.0	2	25.0	1	14.3
Total....	4,662	2,985	64.0	1,676	36.0	733	15.8	538	17.6	257	15.3

* The key to the percentages given for table 1 also applies to table 2.

TABLE 2B (Second Five Year Period).—Age and Sex Distribution of White Persons Coming to Autopsy Between July 4, 1940 and July 4, 1945 Whose Cases Were Surveyed in This Study, with the Incidence of Cancers in Each Age Group

Age Group, Yr.	Persons in Group		Males in Group		Females in Group		Persons with Cancer in Group		Males with Cancer in Group		Females with Cancer in Group	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
0-9.....	418	10.1	314	8.4	304	13.8	1	0.24	0	0	1	0.3
10-19.....	58	1.4	32	1.3	26	1.6	3	5.3	1	3.1	2	7.7
20-29.....	114	2.8	51	1.9	63	3.9	5	4.4	4	7.8	1	1.6
30-39.....	213	5.2	119	4.6	94	5.6	27	12.6	10	8.4	17	18.1
40-49.....	273	9.0	217	8.5	156	9.9	64	17.1	33	15.3	31	20.0
50-59.....	706	17.1	471	15.5	235	14.5	100	21.6	109	23.1	31	21.7
60-69.....	968	23.8	690	25.5	278	17.4	268	27.1	175	26.9	93	27.5
70-79.....	931	21.8	600	23.1	331	19.7	190	21.7	131	22.2	65	20.9
80-89.....	324	8.1	203	7.5	121	8.9	61	18.5	34	17.6	27	19.1
90-99.....	26	0.6	12	0.4	14	0.8	3	11.5	2	16.6	1	7.1
Total....	4,131	2,549	61.7	1,582	38.3	758	19.1	499	19.8	299	18.3

Of the 70 cancers whose primary site was not determined, most had a regional distribution but with extensive involvement of several organs, such as stomach, liver and pancreas, the pelvic organs or lymph nodes and adjacent structures of the neck. The progressive increase in frequency of this diagnosis over the 15 year period may indicate a greater reluctance to assign arbitrarily the site of origin in

TABLE 2C (Third Five Year Period).—*Age and Sex Distribution of White Persons Coming to Autopsy Between July 4, 1945 and July 4, 1950 Whose Cases Were Surveyed in This Study, with Incidence of Cancers in Each Age Group*

Age Group, Yr.	Persons in Group		Males in Group		Females in Group		Persons with Cancer in Group		Males with Cancer in Group		Females with Cancer in Group	
(1)	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
0-9.....	212	8.4	190	8.5	222	9.4	2	0.8	1	0.5	1	0.5
10-19.....	21	0.5	15	0.6	18	1.1	2	6.3	2	13.3	0	0.0
20-29.....	67	1.5	39	1.3	38	2.6	8	4.5	1	2.5	2	5.3
30-39.....	130	3.3	60	2.7	69	4.3	21	17.5	9	15.0	12	20.0
40-49.....	272	7.4	174	7.9	98	6.8	32	19.1	29	14.4	27	27.5
50-59.....	451	12.3	297	13.4	154	10.8	102	22.6	66	22.2	26	25.4
60-69.....	900	24.6	581	26.1	319	22.3	240	26.6	162	27.9	78	24.5
70-79.....	968	27.0	606	27.3	361	26.5	217	22.0	136	22.5	81	21.3
80-89.....	469	12.8	285	11.3	217	15.3	101	21.5	87	23.6	44	20.3
90-99.....	41	1.1	22	0.9	19	1.3	11	26.8	7	31.3	4	21.0
Total.....	2,049	1,225	60.9	1,434	30.1	731	30.6	498	29.9	285	20.0

TABLE 3.—*The Twenty-Five Most Common Primary Cancers in Order of Frequency in the Autopsy Series*

Cancers	1935-1950 Total			1935-1949 Period			1940-1949 Period			1945-1950 Period		
	Numerical Order	No. of Cases	Incidence %	Numerical Order	No. of Cases	Incidence %	Numerical Order	No. of Cases	Incidence %	Numerical Order	No. of Cases	Incidence %
Large intestine.....	1	328	2.6	1	100	2.3	1	112	2.7	1	150	2.0
Lung.....	2	242	1.9	2	70	1.5	2	80	1.9	2	99	2.4
Stomach.....	3	213	1.7	3	79	1.7	3	74	1.8	3	66	1.6
Prostate.....	4	149	1.2	4	66	1.0	7	42	1.0	5	61	1.7
Cervix uteri.....	5	131	1.1	5	43	0.9	6	43	1.0	6	45	1.2
Breast.....	6	113	0.9	10	30	0.6	4	45	1.1	7	38	1.0
Bladder.....	7	107	0.9	4	35	1.2	10	30	0.7	12	22	0.6
Esophagus.....	8	104	0.8	7	42	0.9	4	45	1.1	14	17	0.5
Pancreas.....	9	99	0.8	12	27	0.6	9	33	0.8	6	30	1.1
Brain.....	10	96	0.8	9	33	0.7	6	35	0.8	10	20	0.6
Prim. site undet.....	11	70	0.6	16	17	0.4	12	22	0.5	8	21	0.6
Ovary.....	12	67	...	14	30	...	12	22	...	11	25	...
Leukemia (all types).....	12	67	...	10	20	...	15	16	...	13	21	...
Liver.....	14	64	...	14	30	...	11	26	...	16	16	...
Kidney.....	14	64	...	20	12	...	14	21	...	8	31	...
Gallbladder.....	16	60	...	13	24	...	15	16	...	15	16	...
Skin.....	17	60	...	16	17	...	20	21	...	18	13	...
Uterus.....	18	38	...	24	8	...	17	15	...	17	15	...
Pharynx.....	18	38	...	19	14	...	17	15	...	23	9	...
Sarcoma (soft tissues).....	20	35	...	20	12	...	19	13	...	20	10	...
Lymphosarcoma.....	21	34	...	18	10	...	23	8	...	20	10	...
Mouth.....	22	30	...	25	9	...	21	10	...	19	11	...
Larynx.....	23	29	...	20	12	...	23	8	...	23	9	...
Tongue.....	24	28	...	25	7	...	25	7	...	23	9	...
Bile duct.....	25	20	5	...	22	9	6	...

obscure cases, or a greater effectiveness of supportive therapy, permitting more patients with cancers to live until the primary site became overgrown. The twofold increase in cases of this type over the 15 year period must be taken into consideration in evaluating increases or decreases in frequencies of specific neoplasms.

Cancers of the large intestine (carcinomas) were the most common in each five year period surveyed. In this analysis, carcinomas of the rectum and sigmoid colon were included with those of the rest of the large intestine, since these were of similar appearance and since the exact location of the tumor was occasionally difficult to determine. A slight but consistent increase in total frequency was found in successive five year periods. There was not, however, a consistent increase in frequency within comparable age groups in succeeding periods, as shown in table 4.

TABLE 4.—Frequency of Cancer of the Large Intestine in Relation to Age and Sex in Successive Five Year Periods

Age Group, Yr.	1935-1940 Period				1940-1945 Period				1945-1950 Period				1955-1960 Total			
	Males		Females		Males		Females		Males		Females		Males		Females	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0-9.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10-19.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20-29.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30-39.....	1	0.5	1	0.7	2	1.7	1	1.1	2	2.3	0	0	5	1.5	2	0.7
40-49.....	1	0.3	7	2.1	5	2.3	2	0.4	2	1.1	2	2.0	8	1.1	11	2.3
50-59.....	15	2.4	6	2.3	12	2.5	6	2.5	9	3.0	3	1.9	36	2.6	15	2.3
60-69.....	35	3.5	12	3.6	29	4.5	15	3.5	17	2.9	10	3.1	74	3.5	34	3.4
70-79.....	19	3.5	11	4.5	22	3.7	12	3.6	29	4.8	18	4.7	70	4.0	41	4.4
80-89.....	3	2.1	2	2.0	4	2.1	5	3.5	6	2.4	11	5.0	13	2.2	18	3.9
90-99.....	0	0	0	0	0	0	0	0	0	0	1	5.3	0	0	1	2.5
Total.....	67	...	39	...	74	...	28	...	65	...	45	...	206	...	122	...

It is suggested, therefore, that the observed increase in frequency of intestinal cancer of the 15 year period is related to the increasing age of the patients examined, since intestinal cancer has a higher incidence in the older age groups.

Carcinoma of the lung, the second most common cancer, occurred 5.7 times more often in males than in females. There was a progressive increase in frequency in the three five year periods surveyed, and, as shown in table 5, there was an almost consistent increase within each age group in the successive five year periods. In both males and females the greatest frequency was found in the sixth decade, and no case was observed in the 82 persons of the survey dying after the age of 90 years. It is improbable that improvements in postmortem diagnosis over 15 years could have produced the increase, for cancer of the lung seldom presented a difficult problem at postmortem examination. These data

thus support other observations³ that the frequency of carcinoma of the lung has increased independently of the aging of the population. The sex ratio in our series was much lower than that reported by Wynder⁴ at Barnes Hospital, St. Louis, where it was 18 to 1 in favor of the male. Factors influencing the selection of patients at a private hospital may help to account for this difference.

Carcinoma of the stomach was the third most common of the cancers found, and the frequency remained about the same in each five year period, despite the advancing age of the patients examined. Moreover, it dropped from second to fourth position in the successive five year periods, having been exceeded in frequency by cancers of the lung and the prostate. Steiner⁵ found that gastric cancer was the most common

TABLE 5.—Frequency of Cancer of the Lung in Relation to Age and Sex in Successive Five Year Periods

Age Group, Yr.	1935-1940 Period				1940-1945 Period				1945-1950 Period				1955-1960 Total			
	Males		Females		Males		Females		Males		Females		Males		Females	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
0-9.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10-19.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20-29.....	1	1.2	0	0	0	0	0	0	0	0	0	0	1	0.6	0	0
30-39.....	1	0.6	0	0	2	1.7	0	0	2	2.3	0	0	5	1.3	0	0
40-49.....	6	1.8	0	0	4	1.8	2	1.3	6	2.4	0	0	10	2.2	2	0.4
50-59.....	28	4.5	4	1.5	22	4.7	0	0	26	8.4	1	0.6	66	4.5	5	0.9
60-69.....	21	2.9	2	0.6	29	4.5	2	0.6	23	8.7	3	0.9	62	4.2	7	0.7
70-79.....	7	1.3	0	0	15	2.3	2	0.6	14	2.9	4	1.0	40	2.3	6	0.8
80-89.....	0	0	0	0	2	1.0	0	0	6	2.4	3	1.4	8	1.4	3	0.6
90-99.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total.....	64	...	6	...	74	...	6	...	81	...	11	...	219	...	28	...

malignant tumor in Caucasoids of Los Angeles County examined post mortem in the period 1918 to 1947, and that the peak incidence occurred at an earlier age than in our series. It is possible that comparative data adjusted to the 15 year period of our survey would have shown a closer correlation with our observations. On the other hand, geographic factors may have been responsible for the difference in incidence of gastric cancer in the two areas surveyed. Some of our cases in which the primary site was undetermined may have been of gastric origin, but their total number could not have brought the incidence of gastric cancer in our series to the level reported by Steiner, and could not have altered the relative decrease in numerical order of gastric tumors.

3. Ackerman, L. V., and del Regato, J. A.: *Cancer: Diagnosis, Treatment, and Prognosis*, St. Louis, C. V. Mosby Company, 1947.

4. Wynder, E. L.: *Washington Univ. M. Alumni Quart.* **13**:159-161, 1950.

5. Steiner, P. E.: *J. Nat. Cancer Inst.* **10**:429-437, 1949.

Carcinoma of the prostate showed an abrupt rise in incidence in the last five year period, both relative and absolute, and within comparable age groups, as shown in table 6. The observed rise in the last period of the survey may in part have been due to more thorough routine examination of the prostate post mortem, stimulated by the reports of Rich ⁶ and Moore ⁷ on the frequency of small prostatic carcinomas. The possibility that this tumor was increasing in true frequency is by no means excluded.

Carcinoma of the cervix uteri was the most common cancer of women. It occurred in 131 patients, or 2.8 per cent of the female patients examined, and constituted 15.3 per cent of cancers in patients of this sex. Although this frequency is lower than that reported by Pack and LeFevre ⁸ for the Memorial Hospital for the Treatment of Cancer and

TABLE 6.—Frequency of Cancer of the Prostate in Relation to Age in Successive Five Year Periods

Age Group, Yr.	1935-1940 Period, Cases		1940-1945 Period, Cases		1945-1950 Period, Cases		1935-1950 Total, Cases	
	No.	%	No.	%	No.	%	No.	%
0-9.....	0	0	0	0	0	0	0	0
10-19.....	0	0	0	0	0	0	0	0
20-29.....	0	0	0	0	0	0	0	0
30-39.....	0	0	0	0	0	0	0	0
40-49.....	2	0.6	1	0.5	1	0.5	4	0.5
50-59.....	5	0.8	2	0.4	2	0.7	9	0.6
60-69.....	14	1.9	14	2.1	11	1.9	39	2.0
70-79.....	21	3.9	18	3.1	30	5.9	69	4.0
80-89.....	3	8.1	7	3.6	14	5.5	24	4.1
90-99.....	1	12.5	0	0	3	13.7	4	9.5
Total.....	46	...	42	...	61	...	149	...

Allied Diseases, New York, the differences between a municipal and a specialized hospital must be considered. A cancer hospital is more likely to receive patients in whom the diagnosis can be more readily made than in the case of internal cancers. As shown in table 7, there was a nearly consistent increase in frequency within each comparable age group in the three five year periods, and the greatest frequency was in the fifth decade of life. It is difficult to explain the observed increase over the past 15 years of a cancer that is relatively easy to recognize, unless it is due to a true rise in incidence of the neoplasm. The suggestion is offered that the municipal hospitals may be assuming a greater place in the treatment or terminal care of patients with cancer of the cervix.

6. Rich, A. R.: J. Urol. **33**:215-223, 1935.

7. Moore, R. A.: J. Urol. **33**:224-234, 1935.

8. Pack, G. T., and LeFevre, R. G.: J. Cancer Research **14**:167-204, 1930.

Carcinoma of the breast was the third most common cancer found in women, having been exceeded in frequency by those of the cervix and the large intestine. The incidence was 2.4 per cent in patients of this sex and 13.2 per cent of all cancers in women of the series were of the breast. The frequency was slightly higher in the second and third five year periods of the survey than in the first. Differences in relative and absolute frequency of breast cancer in our series as compared with those cited by Ackerman and del Regato³ are perhaps dependent on differences in composition of the groups analyzed.

Of the remaining types, or sites of origin, of cancers, it is desirable only to point out that some appeared to have increased in frequency while others remained constant or decreased in the 15 year period covered in this survey. Increases are suggested in the frequencies of pancreatic,

TABLE 7.—*Frequency of Cancer of the Cervix in Relation to Age in Successive Five Year Periods*

Age Group, Yr.	1935-1940 Period, Cases		1940-1945 Period, Cases		1945-1950 Period, Cases		1935-1950 Total, Cases	
	No.	%	No.	%	No.	%	No.	%
0-9.....	0	0	0	0	0	0	0	0
10-19.....	0	0	0	0	0	0	0	0
20-29.....	0	0	1	1.6	2	3.2	3	3.4
30-39.....	6	4.5	6	6.4	6	10.0	18	6.2
40-49.....	18	7.9	10	6.4	10	16.2	38	7.3
50-59.....	11	4.3	10	6.2	9	5.8	30	4.6
60-69.....	6	1.8	9	2.6	7	2.2	22	2.2
70-79.....	0	0	5	1.6	5	1.3	10	1.1
80-89.....	2	2.0	2	1.4	6	2.8	10	2.2
90-99.....	0	0	0	0	0	0	0	0
Total.....	43	...	43	...	48	...	131	...

ovarian, renal and uterine cancers; whereas cancers of the urinary bladder and the esophagus were noted less frequently in successive five year periods.

3. The frequency of malignant neoplastic disease found at autopsy in relation to age.

In table 1 it may be seen that the frequency of all cancers of our series increased with advancing age into the seventh decade. Beyond that age there was in both sexes a decrease in incidence of cancers except in the oldest age group of males. In males 90 or more years of age four of the 11 neoplasms were of the prostate. It may be concluded from these data that, with the exception of cancer of the prostate, the incidence of malignant neoplastic disease does not increase directly and indefinitely as a function of age, and that after the seventh decade of life there is perhaps a lessened chance of bearing a cancer. However, our evidence

would not support the statement that if a person lived long enough he would not have a cancer. Although some types of cancerous lesions, such as carcinoma of the lung or of the cervix, appeared to be limited in their incidence to a certain range of age, some others ascended in frequency beyond such limits.

4. *The age incidence of certain types of cancers found at autopsy.*

In chart 2 the rates of incidence of the six most common types of cancers (large intestine, lung, stomach, breast, cervix and prostate) in relation to age and sex are shown graphically. It will be noted that each of the six types of cancer exhibited a characteristic curve of incidence in the patients of this survey. These data are mostly in agreement with the many observations cited by Ackerman and del Regato,⁸ Moore⁹ and Willis¹ as to the period of life in which each tumor occurs in greatest frequency. In the case of cancers of the breast, it may be of interest that two peaks of incidence were noted, one before and one after the age of the menopause. It is beyond the scope of this report to discuss the factors which may govern the age of greatest incidence of different cancers,¹⁰ but the close agreement found between our data and those of others in respect to age incidence attests to the adequacy of the sample of population surveyed.

Although the number of cancers found in young persons of this survey was too small for tabular analysis, the types were those which are known to be the most common in the early period of life. For example, five of eight cancerous growths in children up to 10 years of age were leukemias; the others were lymphosarcoma and tumors of the brain and the adrenal gland. In patients of the second decade of life there were three examples of soft tissue sarcoma, two of Hodgkin's disease and one each of leukemia, osteogenic sarcoma and tumors of the adrenal gland, the brain, the kidney and the ovary. In the third decade of life the most common cancers were leukemias, and those of the brain, the cervix and the testis.

5. *The frequency of multiple primary cancers in relation to age.*

In our series 85 persons, or 3.7 per cent of those with cancers, bore two or more such primary lesions. In the total series of patients whose cases were surveyed the incidence was 0.68 per cent. The criteria of multiple cancer were essentially those of Warren and Gates,¹¹ who reported 40 instances in 1,078 cancer autopsies, or an identical frequency of 3.7 per cent. In view of their conclusion based on a statistical analysis

9. Moore, R. A.: *Textbook of Pathology: Pathologic Anatomy in Its Relation to the Causes, Pathogenesis, and Clinical Manifestations of Disease*, Philadelphia. W. B. Saunders Company, 1944.

10. Loeb, L.: *Biol. Symposia* 11:197-216, 1945.

11. Warren, S., and Gates, O.: *Am. J. Cancer* 16:1358-1414, 1932.

of a smaller series than that reported here, it appears that multiple cancers occurred more frequently in our series than could be explained on a basis of chance.

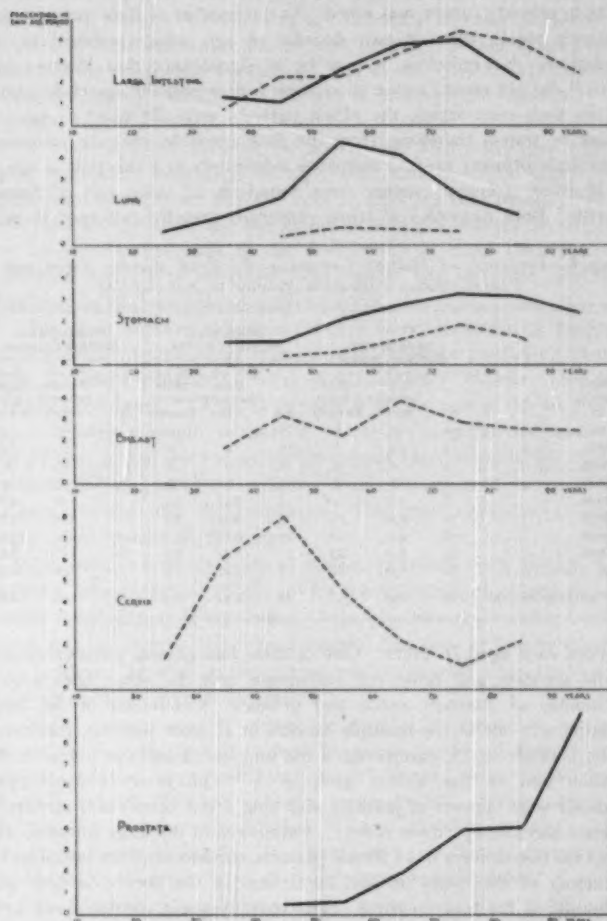


Chart 2.—Graphs of incidence of the six most common sites of primary cancers in males (solid lines) and females (interrupted lines) of the total autopsy series in relation to age.

The frequency of multiple primary cancers in relation to age and sex in the total autopsy series and in cancer autopsies is shown in table 8.

It may be seen that there was a progressive increase in frequency with advancing age in both males and females, regardless of whether the frequency was based on all autopsies or on cancer autopsies only. In the 82 patients who died at 90 or more years of age, no instance of multiple primary cancer was noted. As the number of these patients was relatively small, the last two decades of age were combined in the tabulations. Nevertheless, it may be of significance that Warren and Gates¹¹ did not record a case of multiple cancer past the age of 89 years; and in their own series, the oldest patients were 79 years of age. It cannot be stated, therefore, from the data available, that the frequency of multiple primary cancers increases indefinitely as a function of age.

Multiple primary cancers were found in 53 male and 32 female patients. Both examples of triple cancerous growths occurred in male

TABLE 8.—Frequency of Multiple Cancers in the Total Autopsy Series and in Cancer-Bearing Patients in Relation to Age and Sex

Age Group, Yr.	Patients with Multiple Cancers			Males with Multiple Cancers			Females with Multiple Cancers		
	No.	% of Autopsy Series		No.	% of Male Autopsy Series		No.	% of Female Autopsy Series	
		No.	% of Cancer Bearers		No.	% of Cancer Bearers		No.	% of Cancer Bearers
0-9.....	0	0	0	0	0	0	0	0	0
10-19.....	0	0	0	0	0	0	0	0	0
20-29.....	0	0	0	0	0	0	0	0	0
30-39.....	0	0	0	0	0	0	0	0	0
40-49.....	2	0.17	1.0	1	0.14	1.1	1	0.21	0.9
50-59.....	11	0.54	2.5	8	0.58	2.7	3	0.46	2.2
60-69.....	22	0.75	2.9	14	0.71	2.7	8	0.81	3.2
70-79.....	31	1.16	6.3	21	1.31	5.3	10	1.07	5.3
80.....	19	1.06	6.6	9	1.42	7.0	10	2.00	10.9
Total or average..	85	0.68	3.7	66	0.68	3.6	32	0.68	3.55

patients, each aged 76 years. One of these had glioma, adenocarcinoma of the prostate and renal cell carcinoma, and the other bore adenocarcinomas of stomach, colon and prostate. Carcinoma of the large intestine was one of the multiple cancers in 15 male patients, carcinoma of the prostate in 15, carcinoma of the lung in 13 and carcinoma of the stomach and of the kidney each in 9. The most frequent combinations were cancers of prostate and lung (four cases) and cancers of prostate and kidney (three cases). Carcinoma of the large intestine was one of the two cancers in 11 female patients, carcinoma of the breast in 11, carcinoma of the ovary in six, carcinoma of the cervix in four and carcinoma of the lung in three. The most frequent combinations were mammary and ovarian cancers (three cases), multiple cancers of the large intestine (three cases), cancers of the breast and the uterine cervix (two cases) and bilateral cancer of the breast (two cases). There was thus little evidence of more than chance association of the various types of cancers.

SUMMARY AND CONCLUSIONS

A survey was made of the autopsy records of 12,443 white persons who died in the St. Louis municipal and state hospitals in the 15 year period from July 4, 1935 to July 4, 1950 in order to determine the incidence of cancers in relation to age in both sexes, and to observe any changes in incidence within the 15 year period. The age distribution of the cases surveyed closely paralleled that of the total reported mortality of the community, and about 9 per cent of deaths in the community were included in the survey.

A total of 2,322 patients, or nearly one fifth (18.7 per cent) of those examined, regardless of age, bore cancers. The incidence of cancers increased from 16.8 per cent to 20.6 per cent in the three five year periods surveyed, and the increase could not entirely be accounted for by an observed shift in the distribution of cases toward a more advanced age. It is concluded that cancer has increased slightly in incidence in St. Louis in the past 15 years.

The most common types of cancers were, in order of frequency, carcinomas of the following organs: large intestine (including rectum), lung, stomach, prostate, cervix, breast, urinary bladder, esophagus, pancreas and brain. These 10 comprised 66 per cent of the 46 different types or sites of origin of cancers. Increases in actual frequency over the 15 year period were noted in the case of carcinomas of the lung, the prostate and the pancreas; whereas decreases occurred in those of the urinary bladder and the esophagus. The most conspicuous increase occurred in cancers of the lung.

The incidence of all types of cancer increased with advancing age into the seventh decade of life. With the exception of elderly males, in whom the incidence of carcinoma of the prostate continued to increase, there was a decrease in incidence beyond this age. It is concluded that the incidence of total malignant neoplastic disease does not increase indefinitely as a function of age.

Each common type of cancer exhibited a characteristic pattern of incidence, and the ages of highest frequency were as follows: for carcinomas of the large intestine, eighth decade; lung, sixth decade; stomach, eighth decade; prostate, tenth decade; cervix uteri, fifth decade; breast, seventh decade; urinary bladder, seventh decade; esophagus, seventh decade. It is concluded that factors governing the development of certain types of tumors may be independent of aging.

The incidence of multiple primary cancers was 85 in 2,322 cancer-bearing patients, or 3.7 per cent. This incidence is in agreement with observations of others that multiple primary cancers occur more often than can be accounted for by chance. The frequency of multiple cancers increased with advancing age into the ninth decade of life.

EFFECTS OF A HIGH FAT DIET ON THE JOINTS OF AGING MICE

MARTIN SILBERBERG, M.D.

AND

RUTH SILBERBERG, M.D.

ST. LOUIS

IN YOUNG MICE of strain C57 black reared on a diet containing 30 per cent fat, skeletal growth and development were accelerated. The hastening of growth processes was, however, associated with, or followed by, premature skeletal aging. The changes noted in the articular tissues were interpreted as early manifestations of degenerative joint disease.¹ The present report deals with observations extending over the life span of the mouse. The articular changes taking place under the influence of the high fat diet were studied and compared with those occurring spontaneously in mice receiving a stock diet containing 5 per cent fat.

MATERIAL* AND METHODS

Two hundred and fifty male mice of the closely inbred strain C57 black, raised in our laboratory, were divided into two groups, littermates being equally distributed between the two groups whenever possible. The animals of the first group (control animals) were fed a commercial chow.² The animals of the second group (test animals) were, from the time of weaning, kept on a fat-enriched diet composed of the stock ration ground to a meal, with 25 per cent lard³ added. Both diets were given *ad libitum*, with water available at all times.

From the Snodgrass Laboratory of Pathology, Hospital Division, City of St. Louis, and the Department of Pathology, Washington University School of Medicine.

This investigation was supported by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council, by a grant from the Committee on Scientific Research of the American Medical Association and by a research grant from the National Institutes of Health, United States Public Health Service.

1. Silberberg, R., and Silberberg, M.: *Am. J. Path.* **26**:113-132, 1950.

2. This was the purina® laboratory chow known as ration B 2362, made by the Ralston Purina Company, St. Louis.

3. The silverleaf brand, marketed by Swift & Company, was used.

The composition of the control and experimental diets was, as follows:

	Control Diet	Experimental Diet
Moisture, per cent.....	8.90	6.7
Protein, per cent.....	26.15	19.6
Fat, per cent.....	5.35	29.0
Fiber, per cent.....	4.02	3.5
Ash, per cent.....	6.40	4.9
Nitrogen-free extract, per cent.....	45.66	36.3
Calcium, per cent.....	1.17	0.9
Phosphorus, per cent.....	0.87	0.7
Magnesium, per cent.....	0.196	0.147
Iron, parts per million.....	297.0	246.9
Manganese, parts per million.....	100.0	86.0
Copper, parts per million.....	16.8	12.6
Cobalt, parts per million.....	0.14	0.11
Potassium, per cent.....	0.50	0.66
Carotene, parts per million.....	6.0	5.0
Thiamine, parts per million.....	12.66	9.7
Riboflavin, parts per million.....	7.40	5.0
Niacin, parts per million.....	66.6	62.2
Vitamin D, U. S. P., units per Gm.....	4.97	3.75
Vitamin A, U. S. P., units per Gm.....	9.0	6.75

The experimental diet was thus adequate in all respects.⁴ The protein content, 19.6 per cent, is slightly below the minimum requirement, 20 per cent. However, this deficiency of 0.4 per cent should be amply made up by the increased utilization of protein observed in the presence in the ration of large amounts of fat.⁵

The original plan was to sacrifice groups of 25 animals each at 9, 12, 15, 18 and 24 months of age. However, some animals died prematurely or had to be killed because they appeared ill. In addition, with the high fat diet it was difficult to keep mice alive beyond the age of 18 months. Between the ages of 15 and 18 months there often was a sudden loss of weight which continued for some time, so that the animals had to be killed at irregular intervals. This circumstance accounts for the comparatively large number of test animals in the group 13 to 18 months of age. Some animals which had died and whose tissues had undergone autolysis were eliminated. Altogether, the bones of 113 mice fed the regular diet and of 111 mice fed the high fat diet were available for microscopic examination (table 2).

At necropsy, the individual weight was taken, and pathological findings were recorded. Both legs were removed as a whole, and diseased internal organs, as well as pieces of grossly unchanged tissues, were saved. The legs were freed from the soft tissues, and fixed and decalcified in Bouin's solution. Decalcification was accomplished within six to eight days with one change of fluid after three or four days. The bones were then split by a sagittal cut; one half of a leg was dehydrated in the usual manner and embedded in paraffin; another half to be stained for glycogen was dehydrated in dioxane and embedded in paraffin; still another half was kept in 4 per cent formaldehyde solution for other special stains—in particular, fat stains. As a routine, semiserial sections were stained with hematoxylin and eosin.

4. Morris, H. P.: *J. Nat. Cancer Inst.* 5:115-141, 1944.

5. Forbes, E. B.; Swift, R. W.; James, W. H.; Bratzler, J. W., and Black, A.: *J. Nutrition* 32:387-396, 1946.

GROSS OBSERVATIONS

Weights.—Throughout the second year of life the mean weights of the control animals were, on the whole, constant, varying from 31 to 33 Gm.; from the end of the second year of life on, the weights declined steadily. The weight curve of the test animals roughly paralleled that of the controls, disclosing an over-all increase of about 5 Gm. (15 per cent) over the controls.

The peak seen in the curve of the test animals requires some comment: At the age when this high occurred, the majority of the mice fed the high fat diet were dead; the survivors were either animals with exceptionally high weights or animals in which the terminal loss of

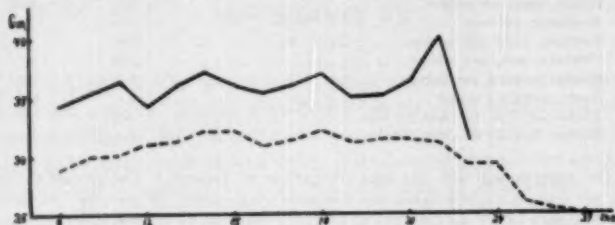


Fig. 1.—The mean weights of control and test mice, respectively. The broken line represents the mean weight curve of control animals; the solid line, that of test animals.

TABLE 1.—Monthly Mean Weights of the Control and Test Animals, with Maximum and Minimum Deviations

Age, Mo.	Control Animals			Test Animals		
	Mean, Gm.	Deviation, Gm.		Mean, Gm.	Deviation, Gm.	
		Maximum	Minimum		Maximum	Minimum
9.....	29.0	4.0	2.0	34.6	5.4	3.6
10.....	30.1	2.9	2.1	35.4	10.6	2.4
11.....	30.2	2.4	1.2	35.3	10.7	4.3
12.....	31.1	3.9	2.1	34.3	20.7	4.3
13.....	31.3	3.7	6.3	35.0	13.0	6.0
14.....	32.2	3.8	1.2	37.0	22.0	8.0
15.....	32.3	0.7	5.3	36.1	28.9	5.1
16.....	31.0	5.0	3.0	35.4	12.6	6.4
17.....	31.4	3.6	3.4	36.0	18.0	8.0
18.....	32.2	2.8	4.2	36.8	22.2	9.8
19.....	31.1	3.9	3.1	35.0	10.0	8.0
20.....	31.4	3.6	3.4	35.1	12.9	6.1
21.....	31.4	8.6	3.4	36.2	14.8	4.2
22.....	31.1	2.9	2.1	40.1	4.9	5.1
23.....	29.4	1.6	3.4	31.1	0.9	1.1
24.....	29.3	2.7	2.3
25.....	26.0	1.0	1.0
26.....	25.4	0.6	0.4
27.....	25.0	2.0	1.0
28.....	26.0	0.5	0.5
29.....	25.0	1.0	1.0
30.....	25.0

weight had not yet taken place. If this small group of selected animals had been excluded, this peak would not be noticeable, and the curve would show instead a gradual decline from the age of 15 months on.

Moreover, the two curves showing the mean weights give only an incomplete picture of the actual conditions without supplementary data on the weight ranges in the control and the test animals, respectively. These ranges appear in table 1 and are graphically demonstrated in figure 2 as deviations from the mean weights.

In figure 2 the area bordered by the two solid lines shows the weight ranges found in the test animals; the area bordered by the broken lines,

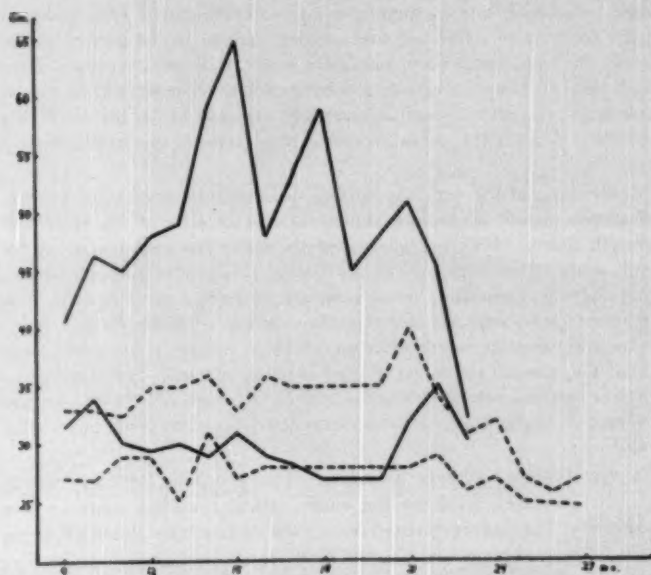


Fig. 2.—The ranges of the weights of control and test mice, respectively. The two broken lines represent the maximum and the minimum weight curves of the controls; the two solid lines, the maximum and minimum weight curves of the test mice.

the weight ranges observed in the controls. In the control group the variations in weight were by far more limited than in the test group. The highest weight ever observed in the control series was 40 Gm.; that is 5 Gm., or about 10 per cent, above the mean found in this group. In the test series the highest weight ever observed was 65 Gm.; that is 28.9 Gm., or about 85 per cent, above the mean in this group. Particularly among the test animals wide variations in weight were noticeable, even within a group of littermates kept in the same cage.

Findings at Necropsy.—In those test animals in which considerable overweight had developed, visceromegaly and extensive deposits of adipose tissue were found in the subcutaneous tissue and in the abdominal cavity. Contact roentgenograms disclosed slightly increased density of the bones.

MICROSCOPIC OBSERVATIONS

The criteria used in the following classification of the articular changes are similar to those established previously.^{6a}

No Change.—Starting from the articular surface and proceeding toward the epiphysis three cell layers may be distinguished: the sliding zone, containing one to three rows of undifferentiated spindle-shaped cells, the axes of which are oriented parallel to the surface of the joint; the transitional zone, containing small ovoid cells separated from each other by abundant amounts of homogeneous chondromucoid ground substance; the zone of ossifying cartilage. An osseous lamella of varying thickness delimits the cartilaginous covering from the epiphyseal marrow (fig. 3A).

The cells of the articular surface thus undergo a cycle of growth, development and senescence similar to that of cells of the epiphyseal growth zones. However, whereas in the latter this cycle ceases at the end of the growth period, in the joints it continues throughout life, though at a diminishing and ultimately extremely slow rate.^{6b,c} The ligaments are composed of collagenous stroma containing a few fibrocytes and, near the insertion at the articular surface, some precartilaginous cells. The menisci consist of fibrillar connective tissue, fibrocartilage or hyaline cartilage; the membrana synovialis is composed of loose vascular connective tissue covered by a continuous single layer of mesothelial cells.

Age Changes.—Slight Changes: The articular cartilage reveals growth processes involving the entire articular surface more or less uniformly. The undifferentiated cells of the surface layer round off, begin to enlarge, proliferate and become more closely packed than ordinarily; mitoses may be found. The hyperplastic processes are particularly striking in the intermediate zone (figs. 3B and C). Occasionally enlarged cartilage cells are surrounded by a halo of basophilic matrix. More often, however, growth processes occur without demonstrable changes in the matrix, at least in sections stained with hematoxylin and eosin.

6. Silberberg, M., and Silberberg, R.: (a) *Am. J. Anat.* **68**:69-95, 1941; (b) *The Effects of Endocrines on Articular Tissues and Their Relation to Ageing Processes*, in *Proceedings of the Seventh International Congress on Rheumatic Diseases*, 1949, Philadelphia, W. B. Saunders Company, to be published; (c) *Growth* **13**:359-368, 1949.

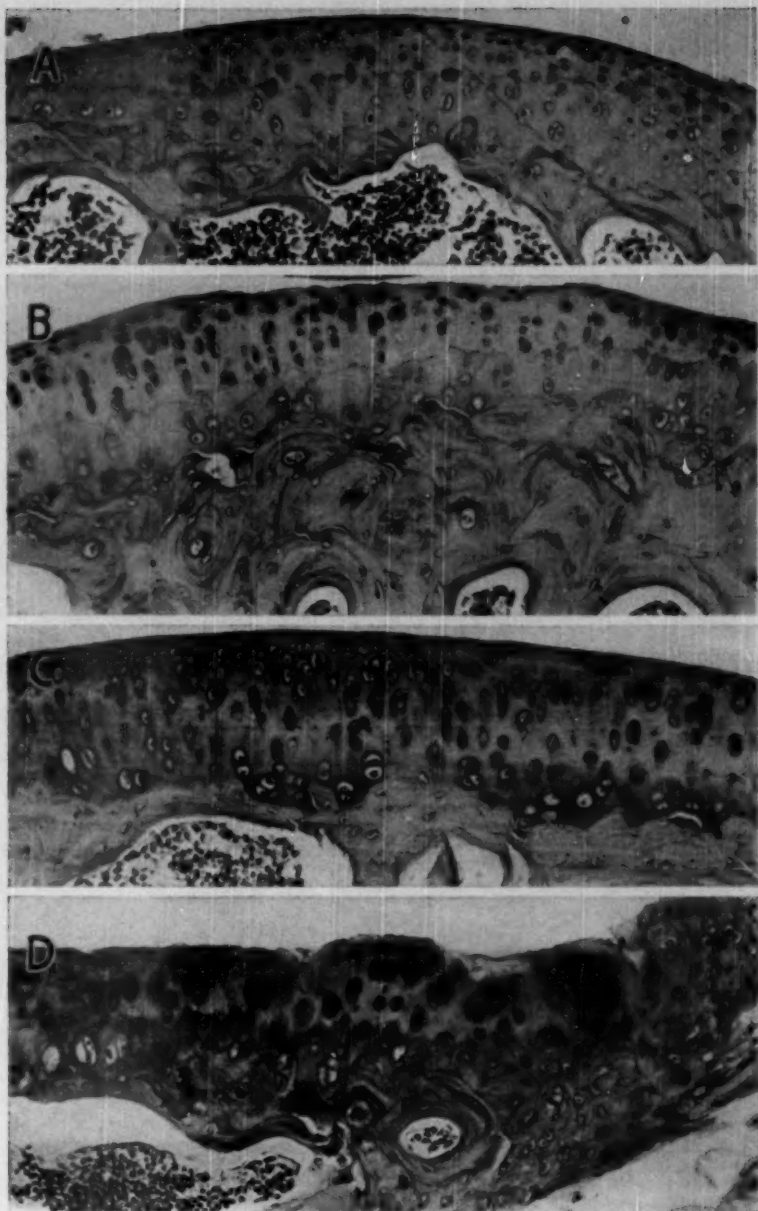


Fig. 3.—A, resting articular surface of the upper end of the tibia; 22 month old control mouse; $\times 200$.

B, slight hyperplasia and hypertrophy of the articular cartilage at the upper end of the tibia; 15 month old control mouse; $\times 200$.

C, moderate hyperplasia and hypertrophy of the articular cartilage at the upper end of the tibia; 12 month old control mouse; $\times 200$.

D, hyperplasia, hypertrophy and marked regressive change in cartilage cells and matrix at the surface of the patella; 9 month old test mouse; $\times 200$.

Increasingly from the age of 12 months on, the ligaments undergo cartilagination; the menisci begin to have centers of marrow and ossify. Moreover, the membrana synovialis may proliferate (fig. 4) and show swelling or fibrinoid change. Proliferation usually sets in near the insertion of the cruciate ligaments, and from here the areolar tissue may spread into the epiphyseal marrow through dilated vascular channels. Thus foci of fibrosis of varying density appear in the epiphysis (fig. 5 *A* and *B*). The changes in the synovialis are not associated with inflammatory processes, and they may occur without changes in the cartilage.

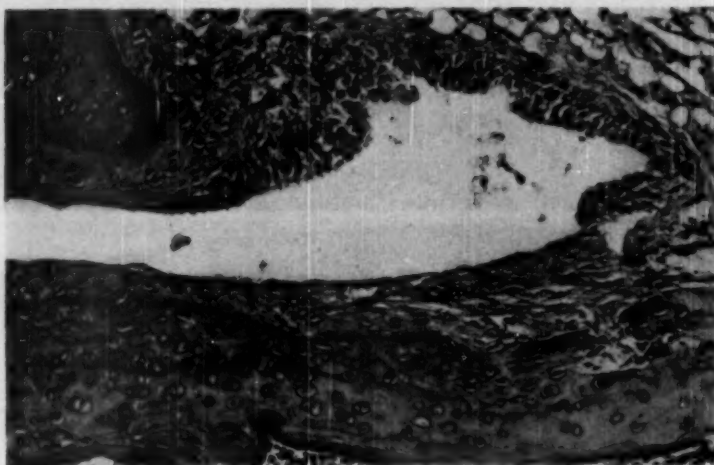


Fig. 4.—Thickened and proliferating membrana synovialis; 22 month old control mouse; $\times 200$.

Advanced Changes: The articular cartilage reveals intensification of growth processes and prominence of regressive changes (fig. 3 *D*). Hypertrophic cartilage cells with pyknotic nuclei and basophilic halos appear in increasing numbers. Unlike the cells of the growing joint which undergo the regular cycle of development, these enlarged cells remain near the surface; furthermore, they do not show the vacuolation typical of differentiating cartilage cells. Focal proliferation of cartilage cells may be observed, leading to the appearance of small clusters adjacent to areas of degeneration. These changes go hand in hand with marked basophilia of the matrix and focal irregularities such as fraying or roughening of the surface, especially near the insertion of the liga-

ments. If these changes are accompanied by alterations in the ligaments or the synovialis, they merge gradually into the picture of osteoarthritis.

Osteoarthritis.—The prominent features are severe distortions of the usual structure of the joint (figs. 5 C to 9). The surfaces of femur, tibia and patella may be affected. The changes vary in kind and show all com-

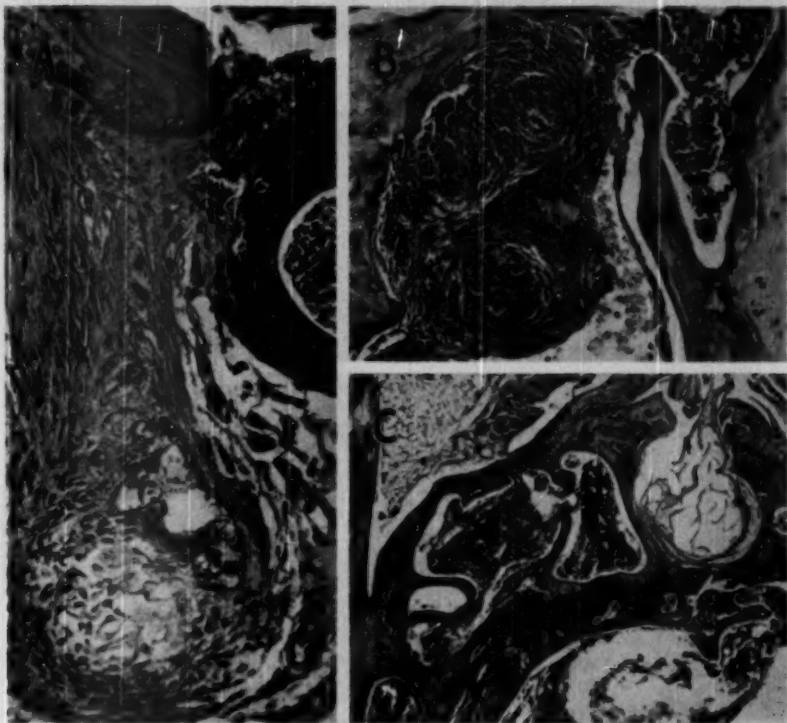


Fig. 5.—*A*, fibrosis and swelling of the membrana synovialis, with connective tissue beginning to grow into the epiphysis of the tibia through the bone of the surface of the joint; 18 month old control mouse; $\times 200$.

B, fibrous replacement of the marrow of the epiphysis of the tibia, representing a stage which is more advanced than that shown in *A*; 18 month old test mouse; $\times 200$.

C, fibrosis and cyst formation in the marrow of the epiphysis of the tibia; the persisting epiphysal plate is visible in the lower part of the photograph; 18 month old test mouse; $\times 45$.

binations of growth processes and regressive changes. The former are hypertrophy and proliferation of the articular cartilage, indicated by the

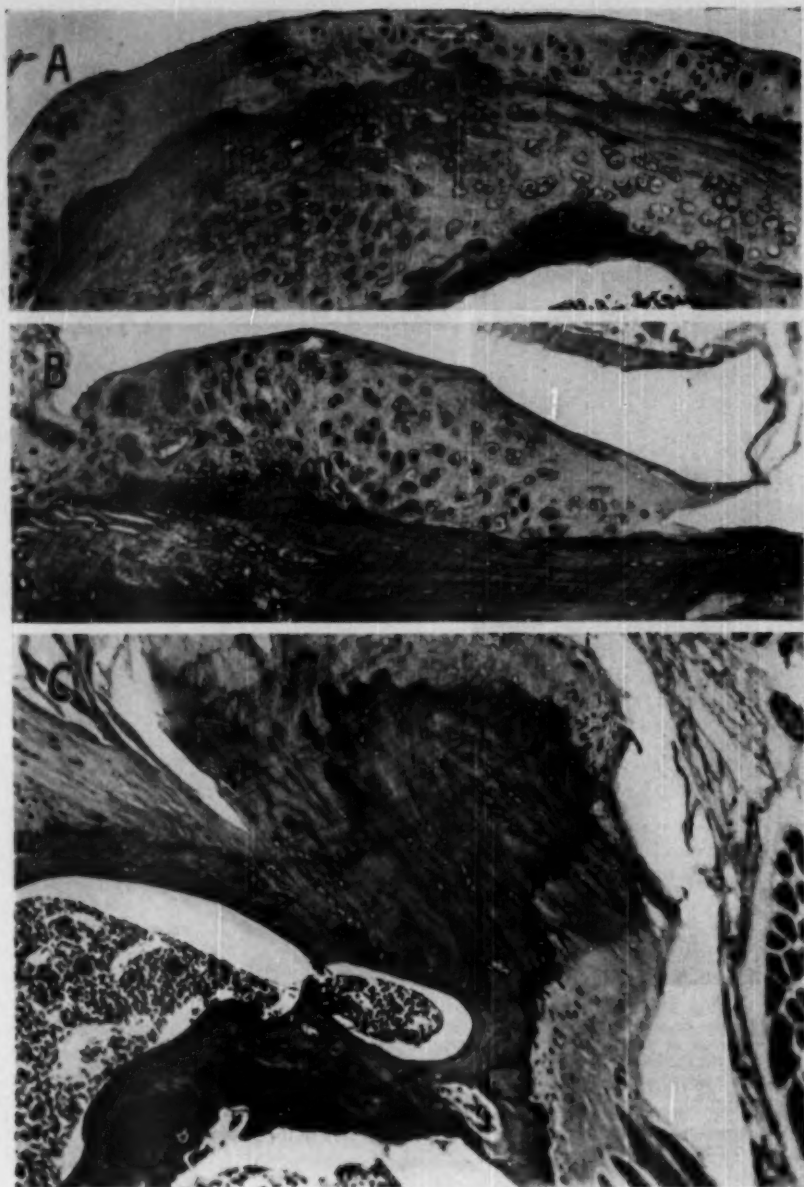


Fig. 6.—*A*, hyperplastic foci in the articular cartilage of the tibia; 18 month old control mouse; $\times 200$.

B, cartilaginous overgrowth at the insertion of a ligament of the tibia; 20 month old test mouse; $\times 200$.

C, bony spur at the articular margin of the tibia arising from ossification of the capsule of the joint; 18 month old test mouse; $\times 200$. Skeletal muscle may be seen at the right; the epiphyseal plate, at the lower left of the photograph.

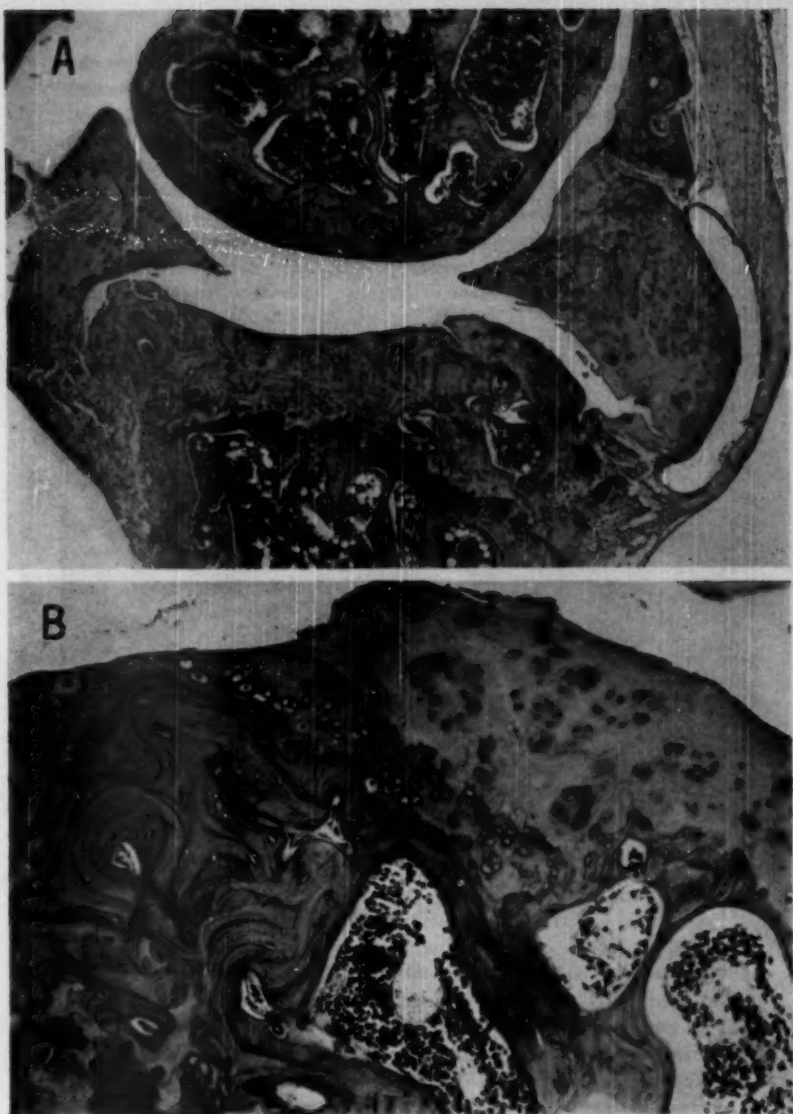


Fig. 7.—*A*, osteoarthritic ulcers at the opposing surfaces of tibia and femur; 18 month old test mouse; $\times 45$. There is condensation of the bone at the floor of the ulcers.

B, part of the ulcer of the tibia shown in *A*; $\times 200$. There is eburnation at the floor of the ulcer and hyperplasia of the cartilage at the margin.

occurrence of incubator capsules and considerable overgrowths. At the same time, cartilage cells are destroyed, and the matrix may be loosened and discloses pronounced pericellular basophilia. Foci of liquefaction, grooves and erosions of the articular surface are common. Loss of cartilage in its entire thickness may cause an ulcer, the floor of which is composed of distinctly thickened sclerotic bone (eburnation). This type of lesion often involves both femur and tibia. Owing to the grinding action of the opposing surfaces, the latter become denuded and thus assume a smooth, polished appearance. The edges of such ulcers usually consist of narrowing tongues of cartilage, and there may be reactive

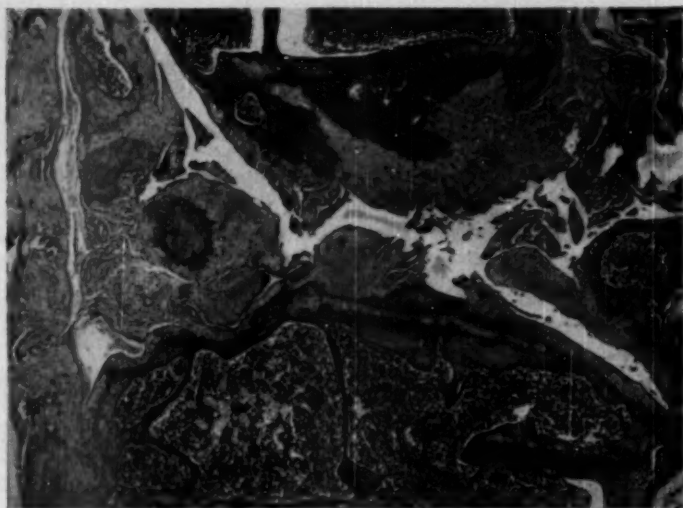


Fig. 8.—Hypertrophic osteoarthritis with marked deformities and overgrowths at the articular surfaces of tibia and femur; 24 month old control mouse; $\times 45$.

hyperplasia of cartilage with formation of irregular prominent outgrowths. Islands of cartilage are attached to the ligaments, or they appear as free bodies in the articular cavity. There is vascularization of cartilage and bone, and at the articular margins, ossification of ligaments with the formation of osseous projections. The synovialis shows proliferation, increased vascularization and fibrosis with infolding or adherence to the surface of the joint. In the ligaments there are advanced fibrinoid change, cartilagination, ossification and sometimes shrinkage. In the epiphyseal marrow, cysts, areas of fibrosis or foci of extravasated blood are noticeable.

Owing to the variety and different combinations of these changes, the microscopic picture of the individual joint varies considerably from that of another. The severe joint lesions of old age are frequently characterized by paucity of cells and predominance of processes of hyalinization affecting areas of the cartilaginous covering as well as the synovialis. The ligaments may be shortened and thickened, and contain foci of



Fig. 9.—Hypertrophic osteoarthritis; 18 month old test mouse; $\times 200$. Marked regressive changes and growth processes are seen at the articular surfaces of the tibia and femur.

hyalinized and ossified cartilage. In these instances one has apparently to deal with "burnt-out" stages of osteoarthritis.

INCIDENCE OF ARTICULAR FINDINGS IN THE VARIOUS AGE GROUPS

The findings in the knee joints are presented in table 2. The animals were divided into the following four age groups: 9 to 12 months; 13 to 18 months; 19 to 24 months; 25 to 30 months. The number of animals

available in each group and their mean ages are shown in vertical columns 3 and 4; the incidence of the findings expressed as number per cent of animals in each age group is given in vertical columns 5 to 8.

At 9 to 12 months of age the knee joints were uninvolved in 22.2 per cent of the controls and in 24.2 per cent of the animals fed the fat-enriched diet. With increasing age the normal joints became fewer in both series of animals. Between 13 and 18 months of age the joints of 11.8 per cent of the controls and of 2 per cent of the test animals were unchanged. Between the ages of 19 and 24 months, 5.6 per cent of the controls had normal joints, and 7.4 per cent of the test animals were free of articular changes, compared with the previous low of 2.0 per cent. Similarly, normal joints were found in 14.3 per cent of the controls living beyond the age of 24 months, compared with the previous low in this group of 5.6 per cent.

TABLE 2.—*The Incidence of Articular Lesions in Various Age Groups*

Age, Mo.	Experimental Group	Mice	Mean Age, Mo.	Percentage of Mice Showing Stated Change			
				No Change	Age Change		Osteo-arthritis
					Slight	Advanced	
9-12	Control	28	10.2	22.2	58.4	11.1	8.3
	Test	23	10.9	24.2	33.5	15.2	27.3
13-18	Control	24	16.8	11.8	49.9	5.9	32.4
	Test	21	16.3	2.0	35.7	19.7	48.6
19-24	Control	26	22.2	5.6	27.7	17.1	56.6
	Test	27	22.0	7.4	0.0	11.1	81.5
25-30	Control	7	27.1	14.3	57.1	0.0	28.6
	Test	0
9-30 combined	Control	113	17.3	13.3	46.0	8.8	31.9
	Test	111	16.1	9.9	17.1	13.5	59.5

Thus among both the control and the test mice the number of animals with normal joints decreased up to a certain age. However, in the oldest surviving individuals of either group the normal joints were somewhat more frequent than before. In mice fed the high fat diet the decline in the number of unchanged joints was faster and the terminal rise less conspicuous than in the controls. Animals which remain free of articular lesions even in old age may represent a selected group of individuals resistant also to other diseases, and therefore surviving the longest.

In the control group the incidence of slight articular changes decreased from 58.4 per cent at nine months to 27.7 per cent at 24 months; in animals surviving this age a higher incidence (57.1 per cent) was again noted. Owing to the small number of animals available in this group, the rather high incidence of 57.1 per cent is probably not truly representative, but it may well indicate a general trend. Again, these animals may belong to the group of selected individuals resistant to degenerative diseases. The mice, therefore, reach an old age before the articular age changes develop, and during the brief remainder of the life

span no further degeneration of the lesions takes place. Under the influence of the high fat diet the incidence of slight articular changes decreased with advancing age. Between 9 and 12 months of age 33.3 per cent of the test animals showed slight articular changes, compared with 58.4 per cent of the control animals. Between 13 and 18 months of age the incidence dropped rapidly, creating an even more marked difference between control and test animals (49.9 per cent of the controls, 15.7 per cent of the test animals). Slight changes were not recorded in 19 to 24 month old animals fed the high fat diet. The significance of the decrease in slight changes with advancing age in control as well as in test animals can be gaged only in connection with the simultaneous rise of the advanced osteoarthritic lesions. This indicates that the age changes are transformed into the severe types of degenerative joint disease with advancing age as well as under the influence of the high fat diet.

There were only slight variations in the incidence of advanced articular age changes with increasing age or with the change in the diet (table 2, column 7). These findings suggest that the advanced age changes represent a transitional stage from which the articular tissues of susceptible animals pass quickly into the osteoarthritic stage, and which, on the other hand, is not reached by animals resistant to articular disease.

Osteoarthritis was observed in a small number (8.3 per cent) of control animals 9 to 12 months of age. However, the incidence of these lesions increased with advancing age until at the end of the second year of life more than one half (55.6 per cent) of animals were thus affected. Of animals living into the third year of life 28.6 per cent had osteoarthritis. However, this percentage should be evaluated with the fact in mind that only few animals survived to this age. At the end of the first year of life osteoarthritis was three times as frequent in animals fed the high fat diet (27.3 per cent) as in the controls (8.3 per cent). At 18 and 24 months of age the incidence of osteoarthritis had increased in the test animals to 68.6 per cent and 81.5 per cent, respectively, compared with 32.4 per cent and 55.6 per cent in control animals of corresponding age. Thus, as had been found in controls, in the test animals osteoarthritis also became more frequent with advancing age.

In horizontal column 5, table 2, the articular changes in the control and test animals are given irrespective of the age of the mice at the time of death. The number of animals showing normal joints was almost the same in both series (13.3 per cent in the controls and 9.9 per cent in the test animals). However, the incidence of age changes as well as that of osteoarthritis varied markedly in the two groups. Of the control animals 54.8 per cent disclosed articular age changes, while 31.9 per cent had osteoarthritis. In the test animals this proportion was practically reversed: 30.6 per cent disclosed articular age changes, while 59.5 per cent had osteoarthritis.

Irrespective of the age of the animals, the incidence of osteoarthritis was thus increased about twofold, an increase which from a statistical point of view is very significant (significant on the 1 per cent level).⁷

THE RELATIONSHIP BETWEEN ARTICULAR CHANGES AND AGE

Table 3 gives the number of animals and their mean ages grouped according to the findings in the joints at the time of death. The mean age of all animals with normal joints was 14.0 months in the control and 13.0 months in the test group.

The mean age of all control mice with articular age changes was 16.2 months and of those showing osteoarthritis 20.2 months. The corresponding ages of the test animals were 13.7 and 17.8 months, respectively. Thus in control as well as in test animals the difference between the mean ages of mice with age changes and mice with osteoarthritis was about the same (four months for the controls and 4.1 months for the

TABLE 3.—*The Relationship Between the Articular Findings and the Mean Age of the Animals*

Experimental Group	Mice	Mice Showing No Change			Mice Showing Age Change			Osteoarthritis		
		No.	% of Total	Age, Mo.	No.	% of Total	Age, Mo.	No.	% of Total	Age, Mo.
Control	113	15	13.3	14.0	68	64.8	16.2	38	31.9	20.2
Test	111	11	9.7	13.0	34	30.6	13.7	66	59.5	17.8

test animals). On the other hand, mice fed the high fat diet showed articular age changes at a mean age of 13.7 months, whereas the mean age of the controls showing articular age changes was 16.2 months. Under the influence of the high fat diet articular age changes were thus advanced by 2.5 months. A similar difference of 2.4 months was observed between the mean ages of mice showing osteoarthritis (17.8 months for the test mice and 20.2 months for the controls). This difference in the acceleration of the onset of osteoarthritis is very significant statistically. However, the high fat diet caused no further acceleration of the course of osteoarthritis beyond the degree to which the preceding age changes had been hastened.

RELATIONSHIP BETWEEN ARTICULAR CHANGES AND WEIGHT

In table 4 the findings in the joints are given in relation to the mean weights of the animals. Animals over 18 months of age and obviously sick animals, as well as animals whose weights had dropped below 25 Gm., were not included in the computation, because unrelated diseases

7. The statistical analysis of these and the subsequent data was made by Prof. V. W. Lemmon, of the department of psychology of Washington University.

or undernourishment are known to influence the course of articular changes.^{2b,c} The maximum weights served as the basis for the tabulation. Consecutive vertical columns show the mean weights of all control and test animals 9 to 18 months old whose joints were unchanged, or disclosed age changes or osteoarthritic lesions.

In the control series the mean weights of animals with normal joints and of those with age changes did not differ much (29.3 and 30.0 Gm., respectively, in the younger group, and 33.3 and 32.9 Gm., respectively, in the older group). In both age groups the animals showing osteoarthritis had higher mean weights than animals showing normal joints or simple age changes: At 9 to 12 months of age the mice with osteoarthritis weighed 33.0 Gm.; that is 3.7 Gm., or about 10 per cent, more than those showing normal joints. At 13 to 18 months of age the mean

TABLE 4.—*Findings in the Joints and Their Relation to the Mean Weight of the Animals*

Age, Mo.	Experi- mental Group	Mice	Mice Showing No Change			Mice Showing Age Change			Mice Showing Osteoarthritis		
			Deviation, Gm.			Deviation, Gm.			Deviation, Gm.		
			Mean Wt., Gm.	Maxi- mum	Mini- mum	Mean Wt., Gm.	Maxi- mum	Mini- mum	Mean Wt., Gm.	Maxi- mum	Mini- mum
9-12	Control	31	29.3	0.7	2.3	30.0	2.0	4.0	33.0	1.0	4.0
	Test	29	33.3	3.3	0.3	33.7	3.3	3.7	40.6	13.4	10.6
13-18	Control	32	33.3	1.7	4.3	32.9	6.1	4.9	34.7	4.3	3.7
	Test	30	32.5	0.5	0.5	35.6	5.4	3.6	41.3	23.8	9.3
9-18 com- bined	Control	63	31.6	3.4	3.6	31.3	7.7	3.3	34.3	4.7	3.3
	Test	59	34.1	7.6	7.1	33.6	5.4	3.6	41.1	23.6	11.3

weight of mice with osteoarthritis was 34.7 Gm.; that is 1.4 Gm., or about 4 per cent, more than those showing normal joints. If all control animals are considered together, irrespective of their age, it is evident that the mean weight of the mice showing osteoarthritis (34.3 Gm.) was 2.7 Gm., or about 8 per cent, higher than the mean weight of those with normal joints (31.6 Gm.). Statistically, this difference is moderately significant (significant at the 5 per cent level).

Unlike the controls, the test animals showing age changes had a higher mean weight than animals with normal joints (35.7 Gm., compared with 33.8 Gm., in the younger group; 35.6 Gm., compared with 32.5 Gm., in the older group). These weight differences were, however, slight as compared with those existing between the test mice showing normal joints and those showing osteoarthritis (33.8 Gm., compared with 40.6 Gm., in the younger group; 32.5 Gm., compared with 41.2 Gm., in the older group; 34.1 Gm., compared with 41.1 Gm. for all mice irrespective of their age). The latter difference is statistically significant above the 5 per cent level.

COMMENT

Articular changes occurring spontaneously with advancing age in the knee joints of mice have been described previously.^{9a} These changes consist of growth and regressive processes in the cartilage, fibrinoid degeneration, metaplastic cartilagination and ossification in the ligaments, and proliferation and hyalinization in the membrana synovialis involving bone and bone marrow. While usually the changes in the cartilage predominate, there are instances in which the synovialis is affected without or with only slight participation of the articular cartilage. A combination of advanced growth and regressive changes of the various articular structures results in lesions which may be considered as an analog of human osteoarthritis.

The incidence, the onset and the severity of these changes vary with sex and strain, and they may be modified by administration of hormones or by removal of endocrine glands.^{9b} In our present series of male mice of strain C57 black reared on a stock diet containing 5 per cent fat, 31.9 per cent showed osteoarthritis and 54.8 per cent slight or advanced age changes, whereas 13.3 per cent remained free of articular lesions, even in old age. Of male mice of the same strain receiving a diet containing 29 per cent fat, 59.5 per cent had osteoarthritis, 30.6 per cent age changes and 9.9 per cent unchanged joints. Thus, under the influence of the high fat diet there was an almost twofold rise in the incidence of osteoarthritis. This increase occurred as the result of a shifting away from simple age changes to the lesions of osteoarthritis. The present series of experiments thus shows a continuation of the trend established in growing mice fed the high fat diet up to 6 months of age: The joints of these animals had undergone premature aging and at the age of 6 months presented changes which in controls do not usually develop before the end of the first year of life.¹

Age changes in the articular tissues may therefore be considered as forerunners and possibly pacemakers of osteoarthritic lesions. On the other hand, the number of mice with unchanged joints was about the same (13.3 per cent in the controls and 9.9 per cent in the test animals) no matter which ration was fed. Thus, a certain number of mice seemed to be resistant to the development of articular changes even in old age and even though the animals were exposed to stimuli which would call forth such changes in predisposed individuals.

The test animals not only had a higher incidence of osteoarthritis but had the disease at an earlier age (17.8 months) than the controls (20.2 months). This age difference of 2.4 months is practically identical with the difference in the ages at which simple age changes were found in the two groups of animals (13.7 months in the test group, compared with 16.2

months in the controls). Thus, in the test mice the mean age at which osteoarthritis was seen was advanced by only as much time as the simple age changes had been. The high fat diet, therefore, does not seem to play a role in the transformation of age changes leading to severe degenerative joint disease, but it apparently exerts its most evident effect on the progress of the early age changes. By accelerating the latter, the high fat diet may render the articular tissues more and prematurely susceptible to other injurious stimuli which contribute to the ultimate production of osteoarthritic lesions. These additional stimuli must be unrelated to the conditions of the present experiment, since a number of control mice likewise presented degenerative joint disease.

In view of the possibility that mechanical factors play a role in the etiology of osteoarthritis⁸ and because overweight may exert excessive mechanical stress on the joints, the relationship between weight of the animals and articular lesions may be briefly discussed. If overweight should actually have any influence on the articular tissues, then not only the degree of overweight but also the length of time during which it is maintained as well as the age at which it occurs might be of importance. Control animals showing osteoarthritis had a mean weight of 34.3 Gm., whereas the test animals with normal joints had a mean weight of 34.1 Gm. Thus, weight as such is not an essential factor in the initiation of the articular changes. Moreover, individuals of the same weight showed different types of articular changes, while animals disclosing similar articular lesions differed widely in their weights. However, within both the control and the test groups osteoarthritis was associated with a tendency toward higher weights. This statistical coincidence was of moderate significance only. It does not necessarily prove an etiologic relationship of overweight and osteoarthritis but may be interpreted as independent responses of body weight and articular tissues, respectively, to the dietary regimen.

The mode of action of the fat-enriched diet on the articular tissues is as yet unknown. One of the major questions arising from the present investigation is the one regarding the specificity of the results. Our present experimental diet contained 6.0 calories per gram, compared with 4.2 calories per gram of the stock diet. A few preliminary observations indicate that, while the ad libitum consumption of individual mice fed the high fat diet varies considerably, the test mice consume at least

8. Payr, E.: *Gelenksteifen und Gelenkplastik*, Teil I, Berlin, Springer-Verlag, 1934. Bennett, G. A.; Waine, H., and Bauer, W.: *Changes in the Kneejoints at Various Ages with Particular Reference to the Nature and Development of Degenerative Joint Disease*, Commonwealth Fund, New York, Oxford University Press, 1942. Hench, P. S.; Bauer, W.; Boland, E. W.; Crain, D. C.; Freyberg, R. H.; Graham, W.; Holbrook, W. P.; Lockie, L. M.; McEwen, C.; Rosenberg, E. F., and Stecher, R. M.: *Ann. Int. Med.* **28**:66-168 and 309-451, 1948.

as many, and usually more, calories than the control animals. Our results may thus be due either to the increased caloric intake as such or to a specific action of the fat, or to both.

It is likewise undecided, whether the skeletal effects of the high fat diet are direct ones acting on cells or matrix or both, or whether they are mediated through changes in the general metabolism. These questions will have to be answered with the help of quantitative and histochemical methods.

SUMMARY

In male mice of strain C57 black fed throughout life a diet containing 29 per cent fat, articular aging was hastened as compared with that of controls fed a stock diet containing 5 per cent fat. Under the influence of the fat-enriched diet the incidence of osteoarthritis was increased two-fold and the onset of the disease significantly accelerated. As the incidence of osteoarthritis increased, that of simple age changes decreased. Articular age changes may, therefore, be considered as precursors of, and possibly pacemakers for, the severe lesions of degenerative joint disease. A number of animals remained free of articular changes even in old age and even if fed the fat-enriched diet. Under the conditions of the present experiment the overweight associated with the consumption of the high fat diet played only a minor role, if any, in the pathogenesis of degenerative joint disease.

IMMUNITY IN SCRUB TYPHUS: RESISTANCE TO INDUCED REINFECTION

JOSEPH E. SMADEL, M.D.

WASHINGTON, D. C.

CAPTAIN HERBERT L. LEY Jr.

LIEUTENANT FRED H. DIERCKS

AND

MAJOR ROBERT TRAUB

MEDICAL CORPS, UNITED STATES ARMY

SECOND attacks of scrub typhus apparently occur with considerable frequency among farmers in certain hyperendemic areas of Japan,¹ but documented cases observed during both episodes are rare. Despite the extensive clinical and laboratory studies that have been made of this rickettsial disease during the last two decades, adequate information is lacking on the degree and duration of the immunity which develops. Both men and animals, after having been experimentally infected with one strain of *Rickettsia tsutsugamushi*, are resistant for some months, at least, to reinoculation of a heterologous strain.² Nevertheless, antigenic heterogeneity among strains of *R. tsutsugamushi* is so pronounced that inactivated vaccines and antiserums prepared against one organism generally fail to protect mice against infection with others.³ These antigenic variations may account for the failure of inactivated scrub typhus vaccines to protect men against field exposure,⁴ and may contribute to the high rate of second attacks of the natural disease in those intermittently exposed to infection.

From the Army Medical Department Research and Graduate School and the Commission on Immunization of the Armed Forces Epidemiological Board.

1. (a) Kawamura, R.: Studies on Tsutsugamushi Disease (Japanese Flood Fever), Cincinnati, University of Cincinnati College of Medicine, 1926. (b) Berge, T. O.; Gauld, R. L., and Kitaoka, M.: *Am. J. Hyg.* **50**:337, 1949.

2. (a) Kawamura, R.; Ito, T.; Nakamura, R.; Kamimura, T., and Sato, I.: *Kitasato Arch. Exper. Med.* **14**:75, 1937. (b) Rights, F. L.; Smadel, J. E., and Jackson, E. B.: *J. Exper. Med.* **87**:339, 1948.

3. Bennett, B. L.; Smadel, J. E., and Gauld, R. L.: *J. Immunol.* **62**:453, 1949. Rights, Smadel and Jackson.^{2b}

4. (a) Card, W. I., and Walker, J. M.: *Lancet* **1**:481, 1947. (b) Smadel, J. E.; Bailey, C. A., and Diercks, F. H.: *Am. J. Hyg.* **51**:229, 1950. (c) Berge, Gauld and Kitaoka.^{2b}

The present investigation was undertaken as part of a series of studies on the problem of immunizing human beings against scrub typhus. This report is concerned primarily with the results of inoculating living *R. tsutsugamushi* in patients whom our group had cared for during a previous attack of proved scrub typhus. After preliminary tests had shown that the experimental disease induced in certain recovered persons could be controlled adequately by specific antibiotic therapy, subsequent tests included control groups of volunteers who had not previously had scrub typhus. The latter groups also served as controls for tests in which the disease experimentally induced in nonimmune volunteers was suppressed by chemoprophylaxis.⁵ Most of the work described at this time was carried out at the Institute for Medical Research, Kuala Lumpur, Federation of Malaya.

METHODS

Preparation and Standardization of Living Attenuated Rickettsial Vaccine.

An egg-adapted line of the Gilliam strain of *R. tsutsugamushi* was selected for the inoculation of all but one of the volunteers. This line had been through 113 yolk sac passages and had lost much of its original capacity to induce lethal disease in mice. Infected yolk sac material was lyophilized in a special buffer-sucrose solution⁶ and standardized at the Army Medical Department Research and Graduate School two to five months prior to being used in volunteers in Washington or Kuala Lumpur; during the interval it was stored at refrigerator temperatures. The inoculum for each experiment with volunteers consisted of rehydrated lyophilized material from several ampuls, which was diluted with buffer-sucrose solution so that 0.1 cc. contained an estimated 10 to 100 minimal infectious doses (MID₅₀) for mice. The rehydrated material used for volunteers was titrated intraperitoneally in mice by the usual method, at the same time that it was being injected into volunteers. The Gilliam strain killed mice only in low dilutions; hence, in order to determine the infectious titer of the material, it was necessary to challenge all surviving titration mice with approximately 10 minimal lethal doses (MLD₅₀) of a virulent strain of *R. tsutsugamushi*. In calculating the MID₅₀, all mice which succumbed to the Gilliam strain or which survived challenge with the lethal strain were considered as infected in the original titration. The calculated number of MID₅₀ in a portion of the material actually injected into volunteers is recorded in this report.

A second strain of *R. tsutsugamushi* was used for the infection of one volunteer. This organism (V-10) had been recovered in January 1947 from the blood of this particular volunteer during a scrub typhus infection which had resulted from laboratory work with the Volner strain of *R. tsutsugamushi*. The agent, after two mouse passages, was stored frozen until the spring of 1950; then, after one additional passage in this host, it was transferred to the yolk sacs of embryonated eggs. A frozen suspension of the rickettsias of the second yolk sac passage was diluted to 10^{-8.20} and 0.1 cc. of this was inoculated in the volunteer. The lethal titer of this same inoculum, determined simultaneously in mice, was 10^{-6.7}; thus, the calculated number of mouse MLD₅₀ which this volunteer received was 28.

5. Smadel, J. E.; Ley, H. L., Jr.; Diercks, F. H.; Traub, R.; Tipton, V. J., and Frick, L. P., to be published.

6. Smadel, J. E., and Jackson, E. B.: To be published.

Selection of Volunteers.—Three groups of volunteers were accepted in the present studies. One group included three American adults who had suffered from scrub typhus 1½ to 3½ years previously. Two of these had contracted the disease as a result of laboratory exposure and one in the course of chemoprophylactic tests in Malaya. The remaining volunteers were Asians (Malay, Indian and Chinese) who lived in the vicinity of Kuala Lumpur. The Asians fell into two distinct groups, (1) those who had had no history of scrub typhus and (2) those who had served as volunteers in previous chemoprophylactic field trials and had contracted scrub typhus as a result of sitting in the grass in hyperinfected fields near Kuala Lumpur.⁷ Each member of this last group had been shown to have rickettsemia prior to the time his disease was terminated by specific antibiotic therapy. Physical examination, carried out in the manner previously described,^{7a} showed that each of the volunteers was in good health prior to inoculation.

Inoculation and Care of Volunteers.—To produce scrub typhus in the volunteers, 0.1 cc. of a diluted rickettsial suspension containing a known number of MID₅₀ of the Gilliam or the V-10 strain was injected intradermally. In the large tests the inoculations were carried out rapidly, members of the control groups being given the injections last, and precautions were taken to maintain the viability of the material during the period. Volunteers were observed twice daily at the Institute for Medical Research, where oral temperatures were recorded and the sites of inoculation examined. No restriction was placed on the movement of the volunteers except that they remain in the city and its environs and avoid those localities where scrub typhus was known to be endemic. Incidentally, because of a drought, the cases of naturally occurring scrub typhus were rare during this time even in those hyperendemic areas which generally provided a relatively constant flow of patients. Oral temperatures below 100 F. were considered normal for our ambulatory volunteers in Kuala Lumpur, which is in the third degree latitude north of the equator.

All volunteers who had temperatures of 100 F. or above on two successive occasions were immediately hospitalized. Those in whom the usual clinical signs and symptoms of scrub typhus developed were promptly given specific antibiotic therapy. Those who received chloramphenicol or aureomycin responded in the usual manner,⁸ becoming afebrile in 12 to 36 hours; a few who were treated with terramycin alone responded satisfactorily and are discussed in detail elsewhere.⁹ The patients were maintained in the hospital for at least 10 days following deferescence, after which they returned twice daily to the Institute for Medical Research for an additional two or three weeks of observation.

The clinical and laboratory procedures employed in this study were essentially identical with those previously described in detail.^{8a}

RESULTS

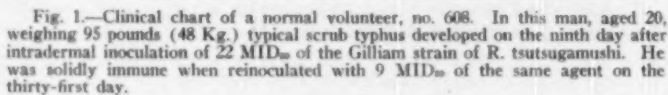
Experimental Scrub Typhus in Normal Volunteers.—Experimental scrub typhus had been induced in normal volunteers in earlier work in

7. (a) Smadel, J. E.; Traub, R.; Ley, H. L., Jr.; Philip, C. B.; Woodward, T. E., and Lewthwaite, R.: *Am. J. Hyg.* **50**:75, 1949. (b) Smadel, J. E.; Traub, R.; Frick, L. P.; Diercks, F. H., and Bailey, C. A.: *Ibid.* **51**:216, 1950.

8. (a) Smadel, J. E.; Woodward, T. E.; Ley, H. L., Jr., and Lewthwaite, R.: *J. Clin. Investigation* **28**:1196, 1949. (b) Smadel, Bailey and Diercks.^{4b}

9. (a) Bailey, C. A.; Ley, H. L., Jr.; Diercks, F. H.; Lewthwaite, R., and Smadel, J. E.: To be published. (b) Smadel, J. E.; Jackson, E. B., and Ley, H. L., Jr.: *Ann. New York Acad. Sc.* **53**:221, 1950.

The disease induced in normal human beings by inoculation of small numbers of active *R. tsutsugamushi* will be discussed first in order to



simplify the comparison of the resultant illnesses in susceptible and partially immune persons. Fourteen volunteers without history of scrub typhus were each given an intradermal injection of approximately 25 MID₅₀ of the Gilliam strain. The course of the disease in these persons was remarkably uniform. On the eighth to the tenth day after inoculation all the volunteers had a febrile illness which subsequently proved to be scrub typhus. The clinical record of volunteer 608, which is reproduced in figure 1, illustrates the typical course.

A summary of the findings in the inoculated normal volunteers is given in table 1. In brief, the sequence of events after inoculation was as follows: A small erythematous lesion appeared within a matter of hours at the site of inoculation and persisted for several days. It began to enlarge on the fourth or the fifth day, and about this time a small papule developed at the center. The lesion increased progressively until specific therapy was instituted. At the onset of fever on the eighth to tenth day the erythematous area had a diameter of about 15 mm. and had a central papule about two-thirds this size and 1 to 2 mm. high. Concurrent with the onset of fever, the patients experienced malaise, severe frontal headache and generally photophobia. Within the first 24 hours physical examination showed little other than injected conjunctivas, definite lymphadenopathy in the axillary area which drained the site of inoculation, and the cutaneous lesion. Specific antibiotic therapy was begun within 48 hours after onset of sustained fever, and defervescence followed promptly. In the majority of the patients the skin lesion remained static for a number of days after treatment; then about the thirteenth day, it began to regress, erythema and edema disappearing rapidly. The site remained discernible, especially in the Asians, for another week as a pale pink, smooth, atrophic area. Three of the 14 dermal lesions progressed to typical necrotic eschar formation during or immediately after defervescence. In these three volunteers the skin lesion healed slowly by granulation, with epidermal coverage by the end of the third week. Photographs reproduced in figure 2 depict various stages of the lesion of one of the volunteers who had a full-blown eschar.

Nine of the 14 patients had recrudescences of scrub typhus on the thirteenth to twenty-second day after inoculation, which was three to 13 days after the beginning of therapy.¹¹ These episodes were promptly controlled by a 3.0 Gm. dose of aureomycin or chloramphenicol. Suppressive therapy which had previously been shown as satisfactory for the prevention of relapses in volunteers who contracted scrub typhus under natural conditions¹⁰ was not attempted in this instance. Convalescence in all of the volunteers was rapid and uneventful.

All 14 of the patients provided positive laboratory evidence of scrub typhus infection with either demonstrable rickettsemia during the acute illness or a significant (fourfold or greater) rise in OX-K antibodies during convalescence. Rickettsemia was demonstrated in 13 and positive Weil-Felix reactions in five. The data for the individual patients are presented in table 1.

10. Footnote deleted.

11. Four of seven patients had a flare-up of the primary lesion at the time of clinical relapse, nine to 13 days after treatment was begun. In these the lesion, which had been regressing, again showed an erythematous area about 10 mm. in diameter, and in two of the four a papule reappeared.

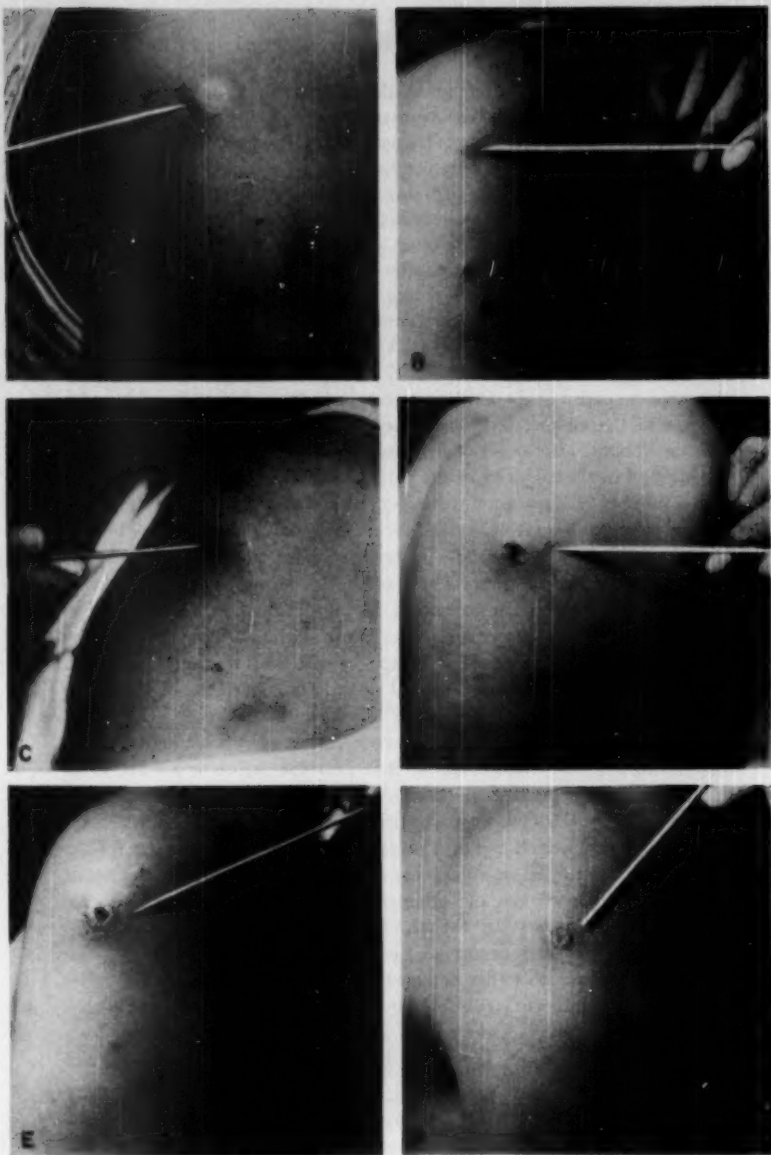


Fig. 2.—Photographs of the skin lesion developing at the site of inoculation of *R. tsutsugamushi* in a normal volunteer, no. 658: *A*, wheal immediately after injection of rickettsias; *B*, small area of erythema and papule five days after injection; *C*, enlarging erythema and papule eight days after injection; *D*, lesion with necrotic center and wide zone of erythema 11 days after injection; *E*, subsiding lesion 16 days after injection and, *F*, lesion after scab detached 26 days after injection.

*Resistance of Recovered Scrub Typhus Patients to Inoculation of
Rickettsias.—Twenty-one persons who had recovered from proved scrub*

TABLE 1.—Summary of Findings in Immune and Nonimmune Volunteers Inoculated with the Gilliam Strain of *R. tsutsugamushi*

Results of Inoculation													
Experiment	Vol. No.*	Previous Disease		Inoculum (Mouse M.D.s)	Clinical Disease	Eschar	Onset of Fever, Days After Infection	Rickettsemia†	Relapse	Total Duration Fever, Days	WF-OX-KI		
		Months Before	Resulted From								Pre	Post	
1	Scrub typhus	CP-25	12	Field	11	+	+	11	+	0	3	0	0
		49	14	Lab.		+	+	10	+	0	4	0	0
2	Scrub typhus	CP-10	22	Field	11	0	0	..	0	0	20
		V-1	16	Field (nat.)		+	+	17	+	0	3	0	20
		122	11	Field		+	+	17	+	0	2	20	40
		44	1	Field (nat.)		0	0	..	0	0	0
3	Scrub typhus	107	10	Field	86	+	+	12	+	0	4	20	20
		111	10	Field		+	+	9	0	0	6	20	20
4-A	Scrub typhus	CP-12	24	Field	22	+	+	11	+	0	5	0	0
		114	17	Field		0	0	..	N. D.	0	0
		124	17	Field		0	0	..	N. D.	0	0
		144	17	Field		0	0	..	N. D.	0	0
4-B	Normal	001	None	22	+	+	8	+	+	6	0	1,200
		003	None		+	+	10	+	+	6	20	20
		005	None		+	+	8	+	0	4	0	20
		007	None		+	+	10	+	+	4	20	20
		006	None		+	+	9	+	0	3	20	20
		013	None		+	+	10	+	+	4	0	20
		015	None		+	+	8	+	+	7	0	20
		016	None		+	+	9	+	0	4	0	20
5-A	Scrub typhus	CP-5	25	Field	9	+	+	12	+	0	2	20	0
		112	18	Field		+	+	11	0	0	2	20	20
		115	18	Field		0	0	..	0	0	20
		120	18	Field		+	+	12	+	0	3	40	40
		430	12	Field		+	+	10	+	0	3	20	20
5-B	Scrub typhus (recent)	CP-10	2	Inoc. (see exper. 2)	9	0	0	..	0	20	40
		122	2		0	0	..	N. D.	0	0
5-C	Scrub typhus (recent)	107	1½	Inoc. (see exper. 3)	9	0	0	..	0	40	40
		111	1½		0	0	..	0	20	20
5-D	Scrub typhus (recent)	006	1	Inoc. (see exper. 4-B)	9	0	0	..	0	20	40
		006	1		0	0	..	0	20	40
		016	1		0	0	..	0	20	0
5-E	Normal	050	None	9	+	+	8	+	+	6	0	200
		054	None		+	+	9	+	0	3	20	20
		055	None		+	+	8	+	+	6	20	20
		056	None		+	+	9	+	0	3	20	20
		057	None		+	+	8	0	+	6	40	100
		058	None		+	+	8	+	+	6	40	20

* The volunteers listed above who had had scrub typhus in the past may be identified in previous publications by the following code: nos. 44, 49 and CP-5 to CP-25 (Smadel, Woodward, Ley and Leethwaite¹⁰); nos. 107 to 120, exclusive of 122 (Smadel, Truh, Frick, Diercks and Bailey¹⁰); nos. 122 and 410 (Smadel, Bailey and Diercks¹⁰). Volunteers V-1 and 44 had experienced previously an additional attack of scrub typhus contracted during field work (see text). All patients were treated with specific antibiotics during the initial attack and the relapse, when it occurred. The total duration of fever includes both the initial illness and the relapse.

† Positive results were obtained from blood specimens drawn during the first three days of disease. Negative results are recorded when the specimens were obtained in the following manner: (1) at a time when other members of the group were shown to have rickettsias in their blood, or (2) during the first three febrile days of illness. N. D. indicates not done.

: WF-OX-K stands for Weil-Felix reaction and OX-K antibodies (see text).

typhus were inoculated intradermally with small amounts of living *R. tsutsugamushi*. The members of this group had contracted their disease in the following manner: 2 from natural field exposure during occupa-

tional duty¹²; 2 from laboratory contact with the organism; 3 from infection induced by injection of Gilliam organisms, and 14 as a result of supervised exposure in hyperendemic areas. All but one of the 21 volunteers were inoculated with the Gilliam strain of *R. tsutsugamushi*; four received injections on two occasions; hence, the number of recovered volunteers listed in table 2 is 24. The single exception (volunteer V-10, a laboratory worker) received the same strain of rickettsia which had been recovered from his blood during the febrile episode 3½ years before; the data on this volunteer are not included in tables 1 and 2 or figure 3.

The incidence of the clinical disease induced in the scrub typhus patients by inoculation of rickettsias is presented in table 2. Among the 16 who had recovered 11 to 25 months prior to inoculation, 11 underwent another attack of scrub typhus. Thus, almost three quarters of this group was not solidly immune to the agent. Eight volunteers who had recovered from scrub typhus which began one to two months before inoculation were all solidly immune.

TABLE 2.—*Disease Induced by Inoculation of Gilliam Strain of R. Tsutsugamushi Into Patients Recovered from Scrub Typhus*

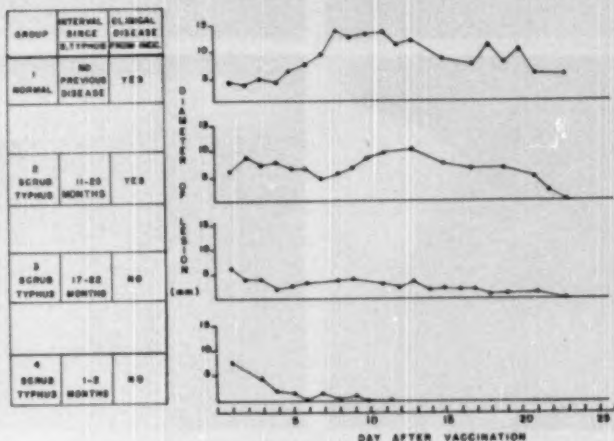
Volunteers	Interval Since Scrub Typhus, Mo.	No. in Whom Disease Developed
8	1-2	0
16	11-25	11
14	No previous disease	14

Those volunteers in whom no febrile illness developed after inoculation of rickettsias showed no untoward signs or symptoms during the period of observation except a local lesion of the skin at the site of inoculation. There was some difference in the course of the cutaneous lesion in the solidly immune group that had had scrub typhus one to two months before and in that which had recovered one to two years previously.

12. The records of these two patients are of particular interest because both had two proved attacks of naturally acquired scrub typhus. Patient V-1, a Dyak engaged as a naturalist for many years in Malaya, had typical scrub typhus in 1932, when he was cared for by Dr. R. Lewthwaite of the Institute for Medical Research. The detailed records of this attack were lost during the Japanese occupation, but there is no doubt about the nature of the illness. He suffered a second episode of scrub typhus in December 1948, when he was observed and treated by our group. At this time rickettsemia was demonstrated but a rise in OX-K antibodies was not found.

Patient 44 was cared for by our group in both of his attacks of scrub typhus; the first of these occurred in May 1948 and the second in March 1950. A summary of the findings in the initial disease is given in table 4 of an article by Smadel and co-workers¹³ and an illustration of the findings in the second episode in figure 4 of another article by Smadel and co-workers,¹⁴ where he is designated as patient N-15.

In both of these resistant groups the initial erythematous reaction averaged about 7 mm. in diameter 24 hours after injection. In the recently recovered volunteers the erythema diminished progressively, disappearing about the fifth day, and was not followed by any detectable local changes (see figure 3). In the second group of immune volunteers, those recovered for one to two years, lesions developed which persisted for about three weeks (see figure 3). In four of the five the cutaneous reaction was limited to erythema for the first two weeks and atrophy and scaling during the last week. The other member of the group showed,



GROUP 1 = VOLUNTEERS NO. - 601, 603, 605, 607, 608, 613, 615, 616, 620, 624, 625, 626, 627, AND 628.

GROUP 2 = VOLUNTEERS NO. - 129, 410, 180, GP-8, GP-18, 107, 111, 112, GP-28, AND 49.

GROUP 3 = VOLUNTEERS NO. - 134, 144, GP-10, 119, AND 115.

GROUP 4 = VOLUNTEERS NO. - GP-10, 122, 107, 111, 44, 609, 609, AND 610.

Fig. 3.—Schematic representation of average diameters attained by the dermal lesions on volunteers at the sites of inoculation.

in addition, a small papule, which appeared at the end of the first week and persisted for several days.

The majority of volunteers who had recovered from scrub typhus one to two years previously had a clinical disease following inoculation of *R. tsutsugamushi* which approached that observed in the inoculated normal controls. Indeed, the severity of the onset of the illness, both as regards symptoms and signs, differed in no respect from that of the naturally acquired disease. No effort was made to study the duration of the illness in these patients, since specific therapy was given within 48 hours after onset. It is worthy of note that the incubation period in this group varied considerably, i. e., from nine to 17 days. Since not all the

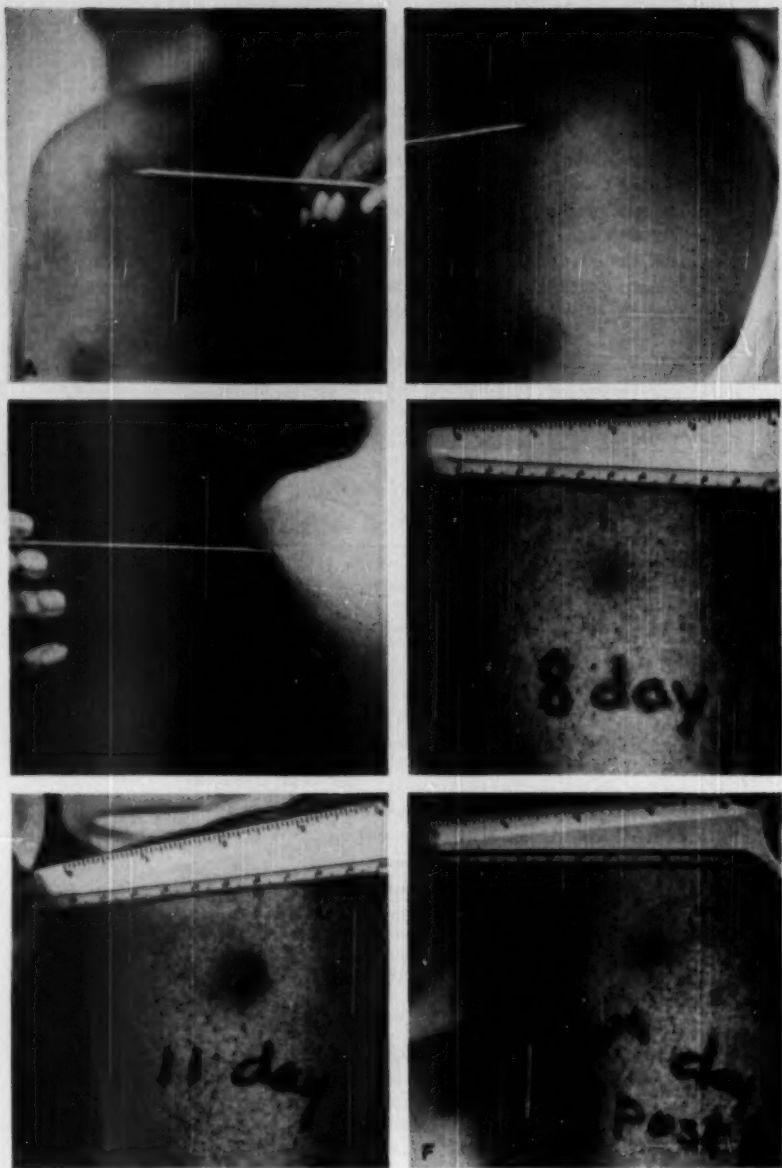


Fig. 4.—Photographs of the skin lesions developing at the sites of inoculation of *R. tsutsugamushi* in two susceptible recovered volunteers. First shown are three photographs of a Malay (volunteer 150): *A*, small area of erythema and papule five days after injection of rickettsias; *B*, enlarging erythema and papule eight days after injection, and *C*, fully developed lesion 11 days after injection. The last three photographs are those of an American (volunteer CP-25): *D*, developing erythema and papule eight days after injection; *E*, enlarging lesion 11 days after injection and, *F*, subsiding lesion 20 days after injection.

volunteers received the same inoculum, definitive conclusions cannot be drawn about the prolongation of the incubation period and the degree of immunity. Nevertheless, it is apparent (see table 1) that the incubation period was consistently longer by several days in the persons who had recovered from scrub typhus than it was in the corresponding normal controls. The lesions which developed at the sites of inoculation in these susceptible volunteers approached those of the normal controls. The data presented in figure 3 show that at the height of the reaction, about the tenth to thirteenth day, the average diameter of the lesion was approximately two-thirds that in the controls. In all the members of the present group papules developed, but in no instance did the cutaneous lesion progress to necrosis and black eschar formation. Photographs illustrating

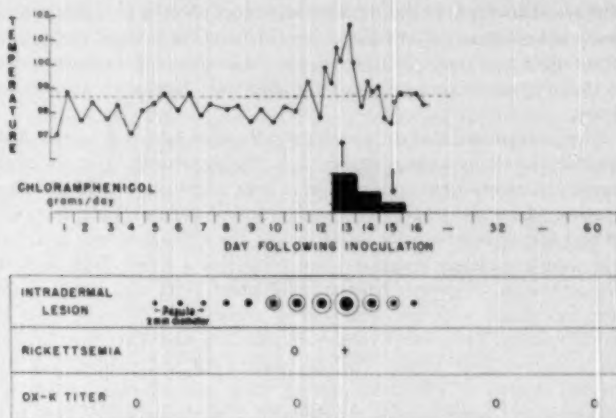


Fig. 5.—Clinical chart of a recovered volunteer, CP-25. This volunteer experienced his initial attack of scrub typhus in June 1948, and subsequently, in January 1950, on the eleventh day following intradermal inoculation of 12 MID₅₀ of the Gilliam strain of *R. tsutsugamushi*, typical scrub typhus developed.

the course of the primary lesion in susceptible recovered Asian and American volunteers are reproduced in figure 4.

The typical course of the experimental disease induced in patients who had recovered from scrub typhus is illustrated in figure 5, which summarizes the data on patient CP-25. Additional information on other patients is given in table 1. All but one of the 11 members of this group had demonstrable rickettssemia during the febrile phase of their disease. It will be noted from table 1 that none of these persons suffered a relapse of scrub typhus, which is in sharp contrast with the 14 normal controls, 9 of whom had recrudescent disease. It should be reemphasized that in neither of the groups was suppressive therapy used to prevent such relapses.

The response of volunteer V-10 deserves special consideration, since he is the only member of the entire group who was reinoculated with the very strain which had been recovered from him during his attack of scrub typhus 3½ years before. The intradermal injection of 28 mouse MLD₅₀ of the homologous strain produced a small erythematous area a few millimeters in diameter, which persisted for several days. No other symptoms or signs developed during the subsequent period of observation.

COMMENT

The present work clearly shows that the majority of persons who have recovered from scrub typhus one to two years previously are susceptible to experimental reinfection with *R. tsutsugamushi*. These observations add credence to the reports that second attacks of tsutsugamushi disease are common under natural conditions.¹ The present findings are discouraging as regards the ultimate success in obtaining a simple method for inducing in human beings a durable solid immunity against this disease.

Any interpretation of the present results must take cognizance of the antigenic variations among strains of *R. tsutsugamushi*. Certain of the present volunteers were known to have been reinoculated with the same organism which was responsible for their initial disease. Volunteers 605, 608 and 666 were originally infected with the Gilliam strain, and these three were completely resistant when reinfected a month later with the same organism. Of greater interest is volunteer V-10, who remained well when inoculated with his homologous organism 3½ years after his original malady. Three other volunteers were known to have been infected with a heterologous strain; CP-25, patient 49 and patient V-1 had provided strains of rickettsias during their first illnesses which had pathogenic and immunogenic characteristics markedly different¹³ from those of the Gilliam strain.¹⁴ Concerning the remaining 14 of the 21 persons studied, no information is available on the relation of the original infecting strain to the Gilliam strain. While rickettsemia had been demonstrated during the first illness of these patients, the agents had not been kept for detailed immunogenic analysis. Other work has shown, however, that marked antigenic variations exist even among strains of *R. tsutsugamushi* isolated in a small geographic area. Thus, the eight strains recovered near Kuala Lumpur, Malaya, from hosts in a field of a few hundred acres fell into four subgroups.¹⁵ The characteristics of

13. Miesse, M. L.; Diercks, F. H., and Danauskas, J. X.: Proceedings of the Society of American Bacteriologists, Baltimore, Maryland Branch of the Society of American Bacteriologists, 1950, pp. 90-91.

14. The rickettsias isolated during the second illness of CP-25 and patient 49, i. e., that which resulted from intradermal inoculation, were characterized and found to be identical with the Gilliam strain. Thus, no evidence was obtained of the resurgence of the original infecting strain during the second episode.

each of these groups differed markedly from those of the Gilliam strain. Hence, it is reasonable to assume that the Gilliam organism, which incidentally came from the Assam-Burma border, was not identical with the agents which originally infected the majority of the volunteers.

The pattern of immunity which begins to emerge as the result of the present work on scrub typhus resembles that of dengue fever. Sabin and his co-workers¹⁵ have demonstrated that human beings who had recovered from one of the three antigenically different strains of dengue virus were resistant to infection with any of the three for a period of months. For a time after this interval the inoculation of a heterologous strain induced a modified clinical disease; somewhat later the heterologous organism induced a classic dengue attack. Even at this stage, a year or so after the original illness, the subject was still solidly resistant to the homologous agent. In the earlier studies of the Japanese,¹⁶ scrub typhus was produced in several hundred persons, consisting of groups of field workers and patients with dementia paralytica, in whom injections of the "attenuated" Pescadores strain were made; the resultant moderately severe disease of two weeks' duration resembled that observed under natural conditions in the Pescadores Islands, where the mortality is low. In 20 of these persons injections of the very highly virulent Niigata strain were made eight days after defervescence of the original disease. Four of the 20 had short, mild febrile episodes, and rickettssemia was demonstrated in these as well as in two others who remained afebrile. These earlier observations of the Japanese, together with our own on patients reinoculated within a month, indicate that a high degree of resistance to homologous as well as to heterologous strains exists during the first month or so after recovery. Kawamura^{16a} reinoculated one of the parietic patients with the Niigata strain three months after this patient had recovered from Pescadores infection and observed a mild disease of several days' duration. Our own experience with persons recovered for one to two years shows that the majority are susceptible to reinfection with known or presumed heterologous strains of *R. tsutsugamushi*. It is to be regretted that more information was not obtained on the duration of resistance to the homologous strain of *R. tsutsugamushi*. Had we originally suspected the outcome of the first tests in this study, we would not have wasted several of our volunteers by challenging them with the Gilliam strain, since their homologous organisms were available in our files. At any rate, in the one instance observed, the volunteer was solidly immune to his homologous organism at the end of 3½ years.

15. Sabin, A. B.: Dengue, in Rivers, T. M.: *Viral and Rickettsial Infections of Man*, J. B. Lippincott Company, Philadelphia, 1948, pp. 445-453.

16. (a) Kawamura, R.; Kashara, S.; Toyama, T.; Nishinarita, F., and Tsubaki, S.: *Kitasato Arch. Exper. Med.* **16**:93, 1939. (b) Kawamura, R., and Ueda, M.: *Ibid.* **16**:183, 1939.

There was a general relationship between the type of skin lesion which developed and persisted at the site of inoculation of rickettsias and the immune state of the volunteer. Although it is difficult to assess the importance of the erythematous reaction noted during the first few days, this appears to have little relation to immunity, since it was present to a similar degree in resistant and in susceptible volunteers. However, beginning with the fifth day, differences were noted in the various groups depending on their susceptibility. Those who were still susceptible, i. e., those in whom clinical disease developed, had local skin lesions which were almost as severe as the controls. Here the progress of the cutaneous lesion was somewhat slower just as the incubation period before onset of fever was slightly longer. On the other hand, relatively little in the way of persistent local reaction was noted in those persons in whom clinical disease did not develop. Nevertheless, even in this resistant group, some variation was noted which was related to the interval since disease. Those recovered within a month or so had no reactions after the fifth day, whereas those recovered a year or so before maintained a small but discernible lesion for about three weeks. It is interesting to speculate on the possibility that the solidly immune, recently convalescent persons suppressed rickettsial growth so completely that no appreciable multiplication occurred at the site of inoculation and that the immune mechanism of the other resistant persons was active enough to prevent generalized infection but not to prevent all multiplication of the organisms in the local lesions.

The persistence of rickettsias for long periods of time in the tissues of certain recovered men and animals is an established phenomenon which undoubtedly has an important bearing on recrudescence illness, such as Brill's disease caused by *Rickettsia prowazeki*, and on the durable immunity which generally exists following most infections caused by this group of agents. The subject is reviewed elsewhere and data are presented on the presence of *R. tsutsugamushi* in the tissue of patients.¹⁷ Suffice it to say here that the agent of scrub typhus was isolated from an axillary lymph node removed from recovered volunteer 144 prior to inoculation with the Gilliam strain. None of the remaining eight volunteers from earlier tests who provided lymph nodes for isolation attempts prior to injection of the Gilliam strain yielded the organism; three of these were subsequently found to be resistant to reinfection, while the rest contracted scrub typhus.

Serums from a number of the present volunteers were tested for complement-fixing antibodies against an antigen prepared from the

17. Smadel, J. E.; Ley, H. L., Jr.; Diercks, F. H., and Comeron, J. A. P.: To be published. Smadel, J. E.; Miesse, M. L.; Diercks, F. H., and Jackson, E. B.: To be published.

Gilliam strain of *R. tsutsugamushi*. The results, which are presented elsewhere,¹⁸ may be summarized as follows: About half of the volunteers who had recovered some time earlier had low titers (1:4 to 1:16) prior to inoculation of the Gilliam organisms, and subsequently practically all had appreciable titers (1:32 to 1:128) irrespective of whether clinical disease developed or not. There was a loose correlation between complement fixation titers and susceptibility to infection. Thus, most of the volunteers with no demonstrable antibody were susceptible, while those with relatively high titers were not. However, certain persons with no antibodies were resistant, and some with titers of 1:4 were susceptible.

An interesting point, on which no definitive data exist, concerns the reason for the resistance to reinfection displayed by the minority of volunteers who had recovered in old cases. Could it be that in these persons the original infecting agents were by chance closely related to the Gilliam strain used for challenge? Perhaps the original organisms were heterologous to the Gilliam strain but were maintained in the tissues of the host and the immunity induced by the continuing suppressed infection was sufficient to resist invasion on injection of any member of the *tsutsugamushi* group. Finally, in a region where scrub typhus is endemic the volunteers may have been exposed to *R. tsutsugamushi* in the interval since the last attack of classic disease, and this may have augmented their waning immunity to a heterologous organism. It is apparent that further studies are indicated.

SUMMARY

In volunteers inoculated intradermally with a few viable rickettsias a disease developed which was indistinguishable from that of volunteers purposefully exposed to infected mites. The course of both types of experimental infection, before it was terminated by specific therapy, closely resembled that of the natural disease. Volunteers were solidly resistant to reinfection with the homologous strain of *R. tsutsugamushi* for one to two months and in one instance for 3½ years. The majority of volunteers infected with a heterologous strain (known or presumed) one to two years after the original disease possessed little, if any, demonstrable immunity.

A general relationship was demonstrated between the state of immunity and the development of a primary lesion at the site of inoculation. Such a cutaneous lesion failed to materialize in the solidly immune, while in the susceptible recovered persons it was quite similar to that in the controls.

In addition to the cooperation of the volunteers who cheerfully experienced the minor inconveniences inherent in their service, aid was received from Dr. J. W. Field, director of the Institute for Medical Research, and the entire staff of the Institute, and from the Government of the Federation of Malaya, particularly the Medical Service.

INCLUSION DISEASE OR GENERALIZED SALIVARY GLAND VIRUS INFECTION

MARGARET G. SMITH, M.D.

AND

FRANK VELLIOS, M.D.

ST. LOUIS

THE DISEASE referred to as "generalized salivary gland virus infection," "inclusion disease" or "cytomegalic inclusion disease" is recognized by the characteristic intranuclear and cytoplasmic inclusions found in cells of various viscera. Intracellular inclusions of this type were first described in 1904, by Jesionek and Kioloemenoglou,¹ who observed them in the kidneys of a stillborn infant. In the same year Ribbert² reported identical inclusions occurring in the kidneys of a newborn infant and in the parotid glands of two older infants. Such inclusions do not occur frequently in the viscera, although the incidence of identical inclusions in the salivary glands of infants and young children, regardless of the cause of death, has been reported as 10 to 32 per cent in routine autopsies.³

For many years the nature of the peculiar cellular changes was unexplained. A number of investigators expressed the opinion that they were protozoan parasites. Others considered the possibility that their intravisceral occurrence was associated with congenital syphilis. At present they are believed to be an expression of a specific viral infection caused by the salivary gland virus.

The morphologic aspects of the inclusions and of the cells in which they occur have been described in detail by Cappell and McFarlane⁴

From the Department of Pathology, Washington University School of Medicine, and St. Louis Children's Hospital.

1. Jesionek, and Kioloemenoglou: *München. med. Wchnschr.* **51**:1905, 1904.
2. Ribbert, H.: *Zentralbl. f. allg. Path. u. path. Anat.* **15**:945, 1904.
3. (a) Lowenstein, C.: *Zentralbl. f. allg. Path. u. path. Anat.* **18**:513, 1907.
(b) Farber, S., and Wolbach, S. G.: *Am. J. Path.* **8**:123, 1932. (c) McCordock, H. A., and Smith, M. G.: *Am. J. Dis. Child.* **47**:771, 1934. (d) Soewadji Prawirohardjo: *Nederl. tijdschr. v. geneesk.* **82**:6218, 1938.

4. Cappell, D. F., and McFarlane, M. N.: *J. Path. & Bact.* **59**:385, 1947.

and by Wyatt and his associates.⁵ We concur with others in the opinion that these cellular changes are of a character that is easily recognized⁶ and is distinct from that seen in other diseases in which intracellular inclusions occur.⁶ The nucleus of the cell is enlarged and the cytoplasm increased in amount. The great size which may be attained by the cell

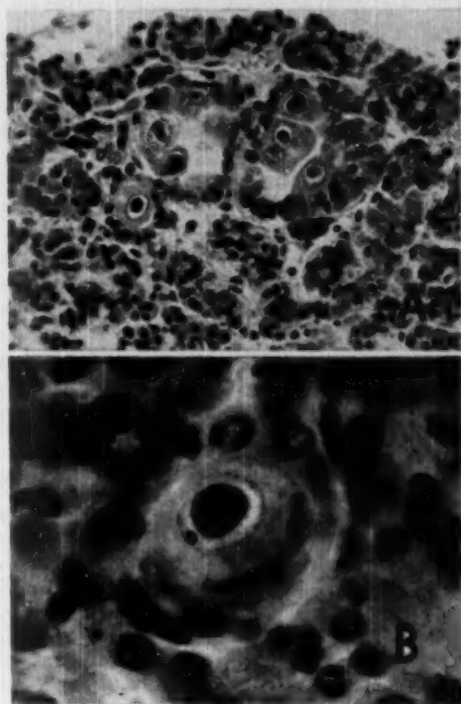


Fig. 1.—*A*, hypertrophied cells of the pituitary gland containing large intranuclear inclusions. Small cytoplasmic inclusions are barely visible. *B*, hypertrophied cell containing a large intranuclear inclusion and cytoplasmic inclusions with crescentic arrangement.

and by the intranuclear inclusion is a conspicuous feature (fig. 1 *A*). The large intranuclear inclusion is surrounded by a clear halo, which

5. Wyatt, J. P.; Saxton, J.; Lee, B. S., and Pinkerton, H.: *J. Pediat.* **36**:271, 1950.

6. Goodpasture, E. W., and Talbot, F. B.: *Am. J. Dis. Child.* **21**:415, 1921.

separates it from a distinct nuclear membrane containing one or more dense chromatin masses. At times the nuclear membrane appears wrinkled or partially collapsed. The shape of the inclusion frequently corresponds to that of the nucleus in which it lies. When stained with hematoxylin and eosin the intranuclear inclusion may be acidophilic or it may have a purplish hue. However, the degree of basophilia of the inclusion is never as great as that of the nuclear membrane. The substance of the inclusion may be granular, or it may appear nodular or uneven in density; at times the peripheral zone stains less intensely than the center. Frequently the inclusion appears homogeneous. Its outline may be sharply defined or hazy. The cytoplasm of the cell is acidophilic or amphophilic and may contain small basophilic bodies, which vary in size and number. The basophilic bodies, measuring 2 to 4 microns in diameter, usually occur in only one part of the cytoplasm and are frequently arranged in a crescentic manner near the periphery of the cell (fig. 1B). The cytoplasm of the larger cells usually is vacuolated and the cell contour irregular. The intranuclear inclusion may occur in a cell which does not contain cytoplasmic inclusions; on the other hand, the cytoplasmic inclusions never occur in a cell which contains a nucleus devoid of an inclusion. At times no nucleus can be seen in a cell containing cytoplasmic inclusions, either because the cell is so large that the nucleus is not in the plane of the section or because the cell is degenerated. The inclusions are usually in epithelial cells but may occur in mesenchymal cells.

EVIDENCE FOR THE VIRAL CAUSATION OF INCLUSION DISEASE

Human inclusion disease has not been transmitted to experimental animals, nor has a virus been demonstrated by the inoculation of embryonated eggs or tissue cultures. However, the intranuclear and cytoplasmic inclusions peculiar to this disease are comparable to those associated with known viral infections of man and lower animals and offer reasonable evidence for the presence of a filtrable virus.

An analogy between the latent disease in the salivary glands of infants and young children and the salivary gland virus disease of rodents gives further evidence for the viral etiology of the disease in man. In many rodents (guinea pigs,⁷ rats,⁸ mice,⁹ hamsters⁹ and moles¹⁰) and also in monkeys, *Cebus fatuellus*,¹¹ inclusions occur in the salivary glands which are similar in all respects to those occurring in the salivary glands of infants and children. These inclusions have been encountered in a

7. Jackson, L.: *J. Infect. Dis.* **26**:347, 1920.

8. Thompson, M. J.: *J. Exper. Med.* **50**:162, 1932.

9. Kuttner, A. G., and Wang, S. H.: *J. Exper. Med.* **60**:773, 1934.

10. Rector, E. J., and Rector, L. E.: *Am. J. Path.* **10**:629, 1934.

11. Cowdry, E. V., and Scott, G. H.: *Am. J. Path.* **11**:647, 1935.

large percentage of the animals of some stock colonies of guinea pigs and mice. In rodents their presence has been associated with a transmissible filtrable virus.¹² However, in each case the virus has proved to be entirely species specific.⁹ Thus, it seems unlikely that the virus producing similar inclusions in the human subject can be transmitted to experimental animals.

The salivary gland viruses of rodents are of low virulence. In most instances no apparent disease is produced in the rodent inoculated with the homologous virus, and inclusions result only in the salivary glands and at the immediate site of inoculation.¹³ However, fatal meningitis with intranuclear inclusions in mononuclear cells of the exudate has been produced in the young guinea pig by intracerebellar inoculation of the homologous virus.¹⁴ Markham and Hudson¹⁵ produced fatal infection in the fetus of the guinea pig by direct fetal or placental inoculation of the virus. Intranuclear inclusions occurred in many organs of the fetus and, in some instances, in the kidneys and lungs of the mother. Rosenbusch and Lucas¹⁶ produced fatal infections in adult guinea pigs, regardless of the site of inoculation, with a strain of virus which appeared to have more than the usual virulence. In young mice a fatal infection has been described following intraperitoneal inoculation of the mouse salivary gland virus.^{17b} In the last instance many intranuclear inclusions and extensive necrosis occurred in the abdominal organs and in the lungs of animals dying four to seven days after the inoculation. Cytoplasmic inclusions were not present. Rosenbusch and Lucas¹⁶ give evidence that in guinea pigs the cytoplasmic inclusions appear in the cells six to 10 days later than the intranuclear inclusions.

There are no reports of fatal spontaneous generalized salivary gland virus disease occurring in rodents, with the possible exception of one by Pappenheimer and Slanetz.¹⁷ They observed an incidental disease associated with widespread intranuclear inclusions in two guinea pigs of a small colony being used for nutritional studies. In their opinion, these inclusions were like those occurring in the salivary glands of guinea pigs. However, they were unable to transmit the disease to other guinea pigs. Markham¹⁸ observed the characteristic inclusions in cells

12. (a) Kuttner, A. G.: *J. Exper. Med.* **46**:935, 1927. (b) McCordock, H. A., and Smith, M. G.: *J. Exper. Med.* **63**:303, 1936. (c) Kuttner and Wang.⁹

13. Kuttner, A. G., and Tung, T'sun: *J. Exper. Med.* **62**:805, 1935.

14. Hudson, N. P., and Markham, F. S.: *J. Exper. Med.* **55**:405, 1932. Kuttner and Wang.⁹

15. Markham, F. S., and Hudson, N. P.: *Am. J. Path.* **12**:175, 1936.

16. Rosenbusch, C., and Lucas, A. M.: *Am. J. Path.* **15**:303, 1939.

17. Pappenheimer, A. M., and Slanetz, C. A.: *J. Exper. Med.* **70**:299, 1942.

18. Markham, F. S.: *Am. J. Path.* **14**:311, 1938.

of the renal tubules in five of 62 guinea pigs of a stock colony. We have recently observed intranuclear inclusions in many organs of several guinea pigs which had been used in a study of the effects of aminopterin,⁸ an antagonist of pteroylglutamic acid (folic acid). Some of these inclusions were identical with those produced by the salivary gland virus. Others were smaller and occurred in nuclei which were not enlarged and did not have a sharply delineated nuclear membrane containing small chromatin masses. In comparing these atypical inclusions with early forms of the intranuclear inclusions which Rosenbusch and Lucas¹⁶ reported in the experimental disease of guinea pigs and with those which we have observed in the experimental disease of mice we were led to believe that they represent developing inclusions of the salivary gland virus type. These few observations offer another analogy of the natural disease of rodents and that of man in that the virus of the guinea pig, like the human virus, may at times produce a generalized infection.

PRESENTATION OF CASES

The characteristic inclusions have been reported in the viscera of infants and young children in 69 instances, but knowledge of the character and of the incidence of the disseminated disease is still incomplete. In 1933 and 1934, 26 instances of this disease were reported by McCordock¹⁹ and by McCordock and Smith.²⁰ We are now reporting 20 additional cases from the same laboratory. Four of these were recognized after further search of the autopsy records in the department of pathology of Washington University, and 16 occurred from 1934 to 1949, inclusive. Three of these cases, in which the patients were infants in the neonatal period, will be presented in detail and the remainder in tabular form.

CASE 1.—A premature white boy, 5 days of age, was admitted to St. Louis Children's Hospital on July 30, 1931 and died six days later. There was no maternal history of syphilis. Two siblings were living and well; a third had died of pneumonia at the age of 18 months. Two days after birth the infant bled profusely from the umbilicus and small purplish spots appeared over the entire body. The umbilical hemorrhage stopped spontaneously but recurred the night before the patient was admitted to the hospital and resisted local measures of control. Physical examination revealed a jaundiced small infant with purpuric spots of generalized distribution. Blood was oozing from the umbilical stump, but there was no frank hemorrhage. The liver and the spleen were enlarged. The laboratory data included a red blood cell count of 1,800,000 per cubic millimeter and a white blood cell count of 21,000 per cubic millimeter, with 20 per cent polymorphonuclear granulocytes, 80 per cent lymphocytes and 3 nucleated red blood cells per 100 white blood cells. A blood film showed a decreased number of thrombocytes. The coagulation time of the blood was 20 minutes; the bleeding time, 35 minutes.

19. McCordock, H. A.: *Proc. Soc. Exper. Biol. & Med.* **29**:1299, 1931-1932.

A transfusion of 70 cc. of citrated blood was given. All signs of hemorrhage disappeared, and the red blood cell count rose to 2,250,000. The infant had attacks of choking during feedings and died during one of these attacks.

At autopsy the body weighed 1,850 Gm. and measured 41 cm. in length. The skin was jaundiced, and there was hemorrhage about the umbilical stump. The left lung was partially atelectatic. The spleen and the liver were enlarged and weighed 20 Gm. and 195 Gm., respectively. The capsule of the liver was thick,

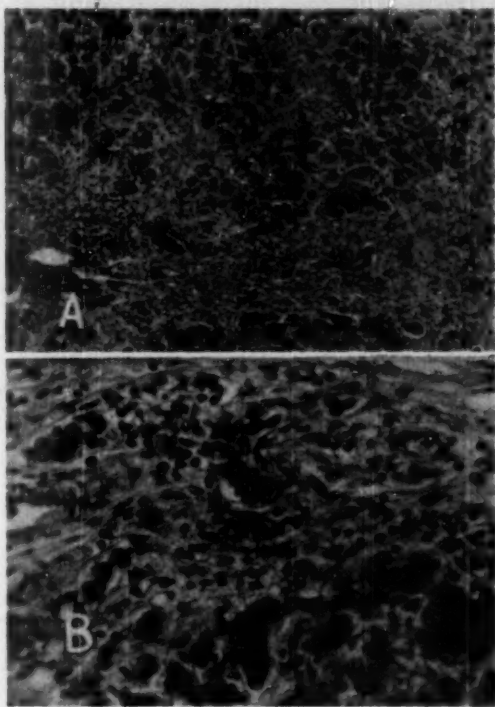


Fig. 2 (case 1).—*A*, liver with periportal fibrosis and with cellular infiltration and intralobular connective tissue isolating groups of intact liver cells. *B*, liver with periportal fibrosis and cellular infiltration. Note the hypertrophied cell of bile duct epithelium with an intranuclear inclusion.

and the surface of the liver was irregular, fissured and dark green. Other organs, including the gallbladder and the extrahepatic bile ducts, were grossly normal. The salivary glands were not examined.

Microscopically, the liver contained small areas of erythropoiesis. There was a marked increase in connective tissue in the periportal zones and within the liver lobules, causing distortion of the structure of many lobules and isolating small

groups of liver cells (fig. 2A). The connective tissue was infiltrated with mononuclear cells. There was an apparent increase in the number of small bile ducts. Bile thrombi were present in the bile canaliculi, and there was bile pigment in Kupffer cells and in liver cells. Inclusions were few and occurred only in the epithelium of bile ducts (fig. 2B). There was erythropoiesis in the spleen, and advanced congestion was present. In the kidneys a large number of the cells of some distal convoluted tubules contained both intranuclear and cytoplasmic inclusions. The involved cells were greatly enlarged, and some of them lay free in the lumens of the tubules. Occasional cells containing intranuclear and cytoplasmic inclusions were seen in the loops of Henle. There were a cellular connective tissue and an infiltrate of lymphocytes about the tubules containing the altered cells. A few foci of erythropoiesis and hemorrhage were present in the kidneys. In the pancreas there were inclusions in the cells of islets, ducts and acini. The interstitial connective tissue was increased in amount and infiltrated with lymphocytes. Slight dilatation of a few small pancreatic ducts was noted. Sections of other organs were not unusual.

Anatomic Diagnosis.—Prematurity; intranuclear and cytoplasmic inclusions of the salivary gland virus type in the liver (epithelium of small bile duct), the pancreas and the kidneys; cirrhosis of the liver; bile thrombi in bile canaliculi; icterus; hepatomegaly and splenomegaly; extramedullary hemopoiesis in the liver, the spleen and the kidneys; hemorrhage about the umbilical stump; petechiae in the renal cortices; focal interstitial fibrosis and lymphocytic infiltration in the kidneys; interstitial pancreatitis, slight; partial atelectasis of the left lung.

CASE 2.—A full term white girl was born on Nov. 20, 1946. The mother was healthy, and her pregnancy had been uncomplicated. She was 24 years of age and had no previous pregnancies. She had never received transfusions of blood or injections of serum. The infant appeared well until one hour after birth, when she became jaundiced and purpura developed. Both increased in intensity before the infant was admitted to St. Louis Children's Hospital. In the hospital the physical findings included jaundice, numerous petechiae of the skin and mucous membranes and a large liver and spleen. Laboratory data included a red blood cell count of 4,000,000; a hemoglobin content of 16.5 Gm. per 100 cc.; a white blood cell count of 23,500, with a differential count of 3 myelocytes, 2 metamyelocytes, 18 stab forms, 36 segmented forms, 35 lymphocytes, 5 monocytes and 1 basophil. There were 42 nucleated red blood cells per 100 white blood cells. The thrombocytes were decreased in number. The icterus index was 250. The bleeding time was more than 30 minutes and the clotting time 2 minutes. The prothrombin time was normal. The test for urinary bilirubin was strongly positive; the stool contained urobilin. The spinal fluid was xanthochromic and contained red blood cells, many of which were crenated. The father was of blood group O, Rh, the mother of group O, Rh, Rh₂ and the infant of group O, Rh.

After the infant was admitted to the hospital, she became more intensely jaundiced, and the purpura increased. A blood culture remained sterile. Diarrhea and edema developed. The child died on the ninth day of life.

At autopsy the body weighed 3,000 Gm. Ecchymoses, petechiae and jaundice of the skin and the mucous membranes were present. The spleen was enlarged (49 Gm.). The liver was enlarged (220 Gm.) and firm. The surface was mottled green and brown and was finely nodular. On the cut surface there were small green and reddish brown nodules measuring 1 to 2 mm. in diameter, separated by a gray network. There were approximately 21 cc. of clear yellow fluid in the peritoneal cavity. The kidneys weighed 12 Gm. each. The cortex of each was bile

stained; the medulla was dark red. In the tentorium cerebelli there were small tears, with a slight amount of fluid blood in the subdural space over both the temporal and the occipital lobes of the brain. Other organs were normal on gross examination. The salivary glands were not examined.

Microscopically, the periportal connective tissue of the liver was increased in amount and was infiltrated with mononuclear cells. The periportal connective

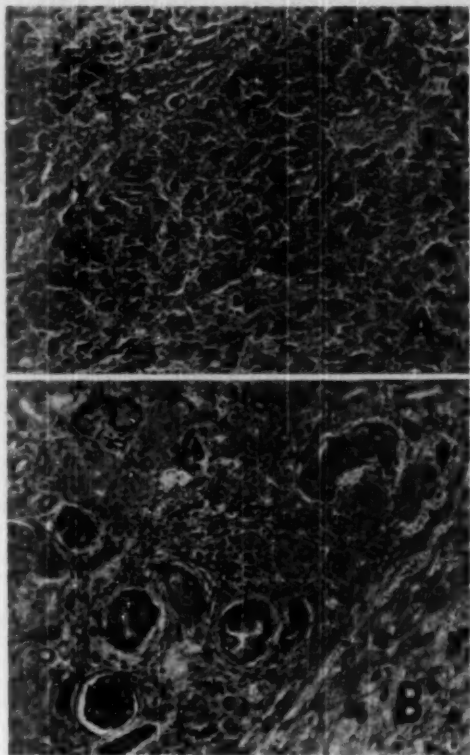


Fig. 3 (case 2).—*A*, liver with periportal fibrosis and intralobular connective tissue. *B*, dilated renal tubules showing hypertrophied cells containing intranuclear inclusions and desquamation of some affected cells. Note focal cellular infiltration.

tissue extended into the periphery of some of the lobules (fig. 3*A*). In many lobules there was a loss of liver cells, so that only collapsed stroma was left. The intact liver cells were vacuolated and contained bile pigment. An occasional liver cell was multinucleated. Bile thrombi were present in the bile canaliculi

and in small bile ducts of the portal spaces. There was bile pigment in the Kupffer cells and in phagocytes in the collapsed stroma where liver cells had been destroyed. A few small foci of erythropoiesis were present. A small number of cells of the intrahepatic bile ducts contained typical intranuclear and cytoplasmic inclusions. In the kidneys, inclusions were numerous in the epithelium of distal convoluted tubules. Some of these tubules, especially those near the capsule of the kidney, were dilated. Many contained desquamated large cells in which the inclusions showed various stages of degeneration (fig. 3B). A few cells in tubules of the corticomedullary junction also contained inclusions. The collecting tubules were distended with granular debris. Some of the granular casts stained orange-red in the manner of hemoglobin. The epithelium of the collecting tubules was flattened and occasionally necrotic. Bile casts were present in tubules of the cortex. There was an interstitial lymphocytic infiltration about tubules in which cells containing inclusions occurred. Small areas of erythropoiesis and hemorrhage were present. These occurred near the blood vessels at the corticomedullary junction. Many cells of islets, ducts and acini of the pancreas contained inclusions. A few small ducts were dilated. There were also slight interstitial fibrosis and lymphocytic infiltration. In the spleen there were a slight diffuse increase of connective tissue and macrophages containing hemosiderin. Erythropoiesis was present. The sinusoids were clearly defined and distended with blood. Intranuclear and cytoplasmic inclusions were present in a large number of the cells of the pituitary and thyroid glands, and a few cells containing inclusions were seen in the rete ovarii. The nucleus of the ovum in an occasional primordial follicle contained an intranuclear inclusion. No intracellular inclusions were seen in sections of the brain. However, in one section which included the wall of the lateral ventricle there was an area of fibrosis and infiltration of histiocytes in the choroid plexus. A few of the histiocytes contained hemosiderin. The ependyma of the ventricle was interrupted at several points by glia extending from the underlying tissue, and the proliferated glial tissue formed the lining of a part of the ventricle. In the adjacent brain tissue the endothelial cells of several small vessels were increased in size and number. There was proliferation of astrocytic glia, as well as an accumulation of small numbers of histiocytes and microglial cells, about these vessels.

Anatomic Diagnosis.—Intranuclear and cytoplasmic inclusions of the salivary gland virus type in the liver (epithelium of small bile ducts), the kidney, the pancreas, the thyroid, the pituitary, and the ovary; cirrhosis of the liver; bile thrombi in small bile ducts and canaliculi; icterus; hepatomegaly and splenomegaly; ascites (20 cc.); a slight degree of extramedullary hemopoiesis in the liver, the spleen and the kidneys; petechiae and ecchymosis in the skin, the pericardium and the renal pelvis; focal interstitial lymphocytic infiltration of the kidneys; nephrosis; subacute ependymitis of a slight degree, and focal fibrosis of the choroid plexus; bilateral tears of the tentorium cerebelli; slight subdural hemorrhage.

CASE 3.—A white girl, 36 hours of age, was admitted to St. Louis Children's Hospital, July 15, 1948. This had been the mother's first pregnancy. She had never received a blood transfusion. The infant was said to have been jaundiced at birth. Soon after delivery a petechial rash developed in the skin. Both the rash and the jaundice became more pronounced. When examined in the hospital the infant was critically ill. She was obviously premature, jaundiced and covered with petechiae and ecchymoses. There was a large amount of dark brown "coffee ground" material coming from nose and mouth. The liver and the spleen were markedly enlarged. Laboratory data included a red blood cell count of 2,500,000; a hemoglobin content of 10.2 Gm.; a white blood cell count of 57,000,

with 2 metamyelocytes, 4 stab forms, 38 segmented forms, 54 lymphocytes and 4 monocytes. There were 144 immature red blood cells per 100 white blood cells. Thrombocytes were almost absent from the blood film. The bleeding time was 15 minutes. The child died while blood was being drawn for culture and typing. The result of the blood culture was subsequently reported as negative. Blood grouping performed later revealed that the father was of group O, Rh, the mother of group A, Rh, Rh, and the patient of group O, Rh, Rh. No Rh antibody of either the direct or the blocking type was present in the mother's blood six weeks after the birth of the child.

At autopsy the body was that of a jaundiced premature infant weighing 2,100 Gm. Numerous petechiae were present in the skin, the mucous membranes and the serous membranes. Fluid blood oozed from nose and mouth. The lungs weighed

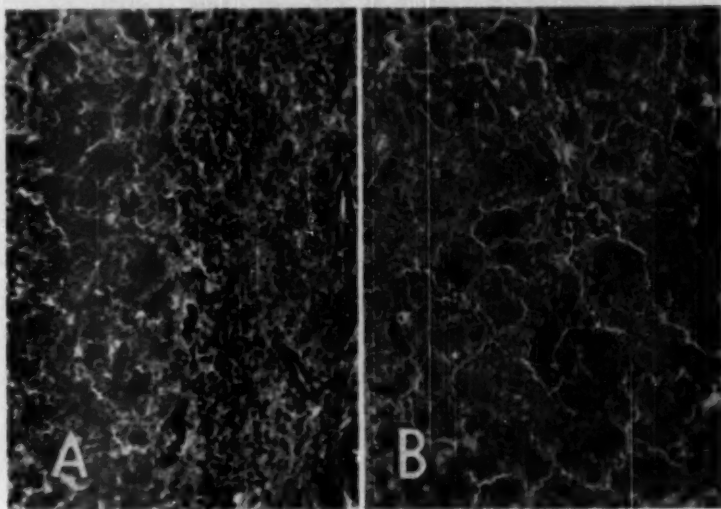


Fig. 4 (case 3).—A, liver with periportal fibrosis and cellular infiltration, and areas of erythropoiesis. B, liver with areas of necrosis of liver cells and multinucleated liver cells.

39 Gm. and contained numerous dark red raised areas. The spleen was large (66 Gm.), firm and dark red. The liver was enlarged, weighing 145 Gm. It was bile stained, and the lobular structure was obscure. The kidneys weighed 12 Gm. each. They were bile stained, and there were areas of hemorrhage in the cortex of each. There was hemorrhage in the tentorium cerebelli, and slight diffuse subarachnoidal and subdural hemorrhages were present.

Microscopically, there were areas of unexpanded or partially expanded lung tissue and hemorrhages in groups of alveoli. Where the alveoli were expanded, the walls were thicker than normal. The increased thickness of the walls was due to an abnormal number of fibroblasts and mononuclear wandering cells. A moderate number of inclusion-bearing cells lay free in the alveoli and occasionally

within the wall of an alveolus. In the liver there was marked erythropoiesis (fig. 4*A*). Necrosis of liver cells and hemorrhage were widespread (fig. 4*B*). There was an increase in the amount of periportal connective tissue, and connective tissue extended into the periphery of liver lobules (fig. 4*A*). Within some lobules and in the periportal connective tissue there was an infiltration of mononuclear cells. There was proliferation of the small bile ducts. Intranuclear inclusions were present in bile duct epithelium and in liver cells near the periphery of the lobules. There were bile thrombi in many bile canaliculi and bile pigment in liver cells. In the pancreas there were numerous intranuclear and cytoplasmic inclusions in cells of islets (fig. 5*A*), ducts and acini, and there were slight interstitial fibrosis and lymphocytic infiltration. In the duodenal mucosa adjacent to the pancreas a

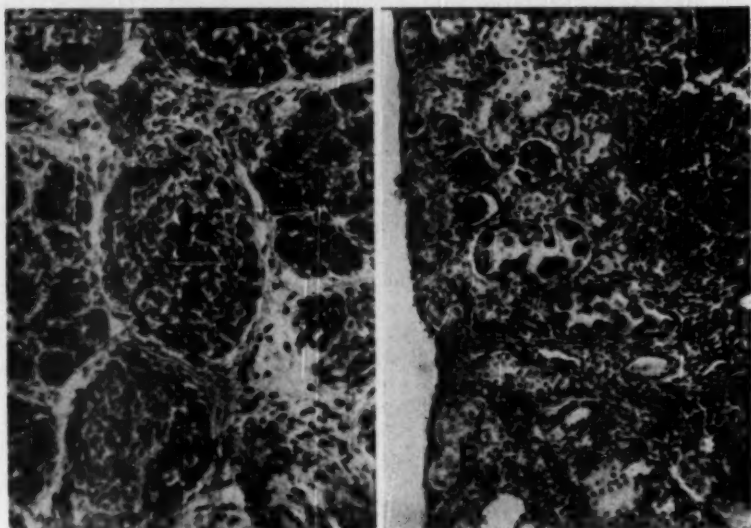


Fig. 5 (case 3).—Pancreas with several hypertrophied cells containing intranuclear inclusions in an islet of Langerhans. There is a slight increase of interstitial connective tissue and cellular infiltration. *B*, dilated renal tubules with hypertrophied cells containing intranuclear inclusions. There is desquamation of some affected cells. Note focal cellular infiltration.

few inclusions were present in the cells of Brunner's glands. There was congestion of the spleen, and erythropoiesis was present. In the kidneys numerous cells of the distal convoluted tubules contained intranuclear and cytoplasmic inclusions (fig. 5*B*). Some of the cells containing inclusions were free in the lumens of the tubules, and the tubules were dilated. An interstitial infiltration of lymphocytes was concentrated about the involved tubules. In addition there were foci of erythropoiesis and hemorrhage about blood vessels in the cortex and at the corticomedullary junction. Intranuclear and cytoplasmic inclusions were found in a large number of the epithelial cells of the pituitary and thyroid glands and in a few epithelial cells of the duodenal mucosa.

Anatomic Diagnosis.—Newborn premature infant; intranuclear and cytoplasmic inclusions of the salivary gland virus type in the liver (epithelium of bile ducts and liver cells), the kidneys, the pancreas, the lungs, the duodenum, the thyroid and the pituitary; hepatitis with necrosis and hemorrhage; icterus; hepatomegaly and splenomegaly; extramedullary hemopoiesis in the liver, the spleen and the kidneys; petechiae and ecchymoses in the skin, the serous and mucous membranes, the renal cortices, the myocardium, the diaphragm and the wall of the gallbladder; altered blood in the stomach and the colon; hemorrhages in the lungs; hemorrhage in the tentorium cerebelli; subarachnoidal and subdural hemorrhages; slight interstitial pneumonia; slight interstitial pancreatitis; interstitial lymphocytic infiltration of the kidneys; partial atelectasis of the lungs.

SUMMARY OF THREE CASES OF INCLUSION DISEASE IN INFANTS
DYING IN THE NEONATAL PERIOD

There is a pronounced similarity in the clinical and anatomic features of the disease occurring in these 3 infants. Jaundice and purpura were prominent in each. A decrease in the number of thrombocytes occurred in each. In two infants there was severe anemia. Enlargement of both liver and spleen was present in each instance, and there was anatomic evidence of hepatic damage. Extramedullary blood formation was prominent in two infants and slight in the third. Focal accumulation of lymphocytes and large mononuclear cells, associated with the occurrence of inclusions, was present in the kidneys. In one instance more extensive renal damage was present. There was slight interstitial fibrosis of the pancreas in each case. Only one of the infants had interstitial bronchopneumonia with cells containing inclusions in the lungs. Subacute ependymitis was present in one case. In none of these infants was there evidence of congenital syphilis. Isoimmunization of the mother in the two instances in which this possibility was investigated seems to have been excluded. Inclusion disease, presumably a generalized salivary gland virus infection, was probably present in each of these three infants at the time of death. All indications are that this infection was the primary cause of death.

SUMMARY OF 17 CASES OF INCLUSION DISEASE
PRESENTED IN TABLE 1

Inclusion disease, as we have observed it in older infants, has been associated with diverse clinical histories and anatomic changes. In some instances another principal disease has been present. Seventeen cases are summarized in table 1. In each of the first two cases presented in this table inclusion disease may have been the principal disease responsible for the infant's death. In the first case the premature infant was born in St. Louis Maternity Hospital and received hospital care in that hospital and in St. Louis Children's Hospital during its entire life of 1½ months. Despite this care he never progressed satisfactorily. There

TABLE 1.—Summary of Seventeen of Twenty Cases of Inclusion Disease in Children

Case and Year	Age of Patient	Organs Showing Inclusions	Clinical History and Findings	Autopsy Observations
1 1935	1½ mo.	Liver, kidneys, pancreas, salivary gland	Seven months premature; frequent bouts of fever during 1st mo.; weight gain very gradual; abdomen frequently distended; 4-6 stools daily; dehydration; difficult breathing and rise in T. on day of death; S. T. S.* negative, but mother had syphilis	Prematurity; blood formation and periportal scarring in liver; sclera jaundiced; bile thrombi in canaliculi; feet of mononuclear cells in kidney; acute interstitial pneumonitis
2 1944	6½ mo.	Lungs, liver, kidneys, pancreas, adrenal, ovary, ileum (large mononuclear cells of mucosa); salivary glands not examined	Persistent severe cough began 2 mo. before death, vomiting and loss of weight; sulfonamide given orally for 1 week beginning 3 weeks before death, followed by oliguria, hematuria, bleeding into areas where subcutaneous fluid was given; convulsions 2 weeks and shortly before death; entered hospital 6 days before death; thrombocytopenia; hemoglobin 11.3 Gm.; WBC 20,000; albuminuria; hyaline and granular urinary casts; T. 38.3-40.5 C. (100.9-104.9 F.); bronchopneumonia; otitis media; convulsions and cyanosis; Kline test negative; blood cultures negative	Interstitial bronchopneumonia; focal atelectasis of lung; acute bronchopneumonia; focal necrosis in adrenal; focal hemorrhage in pleura and gastrointestinal tract; fatty metamorphosis and slight periportal fibrosis of liver; brain and meninges normal; Staph. albus cultured from lungs
3 1930	2½ mo.	Liver, adrenal, ileum (large mononuclear cells of mucosa), meninges; salivary glands not examined	Prematurity; diarrhea, vomiting and loss of weight beginning 15 days before death; 1-12 loose stools daily; pneumonia during last few days; subnormal T.; WBC 15,400, 48% lymphocytes; dextrose solution given into longitudinal sinus	Bronchopneumonia; focal necrosis of adrenal; congestion of liver with central necrosis; bile thrombi in canaliculi; leukocytes about a few meningeal vessels and incursions in endothelial cells; localized hemorrhage in meninges beneath anterior fontanel; no gross intestinal lesions
4 1905	2½ mo.	Lungs, adrenal, ileum (large mononuclear cells of mucosa), salivary gland	Cough and head cold 5 weeks before death; hospitalized 3 days for bronchopneumonia and atelectasis of upper lobe, rt. lung; discharged in good condition but returned 17 days before death with diarrhea and persistent cough; right upper lobe dull to percussion; a few rales in lungs; otitis media; increasing diarrhea not responding to control of diet, intravenous fluid and small transfusions; acute bronchopneumonia 4 days before death	Lipid pneumonia with atelectasis of upper lobe, rt. lung; acute bronchopneumonia; focal necrosis in adrenal cortex; no gross intestinal lesions
5 1923	3 yr.	Colon (large mononuclear cells and capillary endothelium in granulation tissue of mucosal surface), salivary gland	Onset of diarrhea with convulsions, high fever and delirium; no known contact with typhoid fever; spleen palpable; no rose spots; pulse 130; WBC 10,000; Widal test negative; stools negative for pathogens; diarrhea persisting for 2½ mo.; extreme emaciation; distention, abdominal pain and blood in stools at times; otitis media and mastoiditis; died following mastoiditis	Chronic ulcerative colitis; perforation of colon; purulent peritonitis; bilateral mastoiditis; no lesions characteristic of typhoid
6 1940	9 mo.	Ileum and colon (large mononuclear cells of mucosa); mesenteric lymph node; salivary glands not examined	Bronchopneumonia; bilateral otitis media; meningismus 6 weeks before death; severe persistent diarrhea 2 weeks later; failed to respond to sulfadiazine, penicillin or fluid and diet regulation—jaundice occurred following a transfusion but was diminishing when child left hospital 3 days before death; returned in moribund condition 1 hr. before death	Focal enterocolitis; acute inflammation of mesenteric lymph nodes; fatty metamorphosis of liver; acute bronchopneumonia; no gross intestinal lesions
7 1933	3 mo.	Lungs, salivary gland, sections of intestine not available	Well until 8 weeks of age; brassy cough for 3 weeks; diarrhea for 1 week before death, 12 stools per day; otitis media and signs of meningitis 4 days before death	Interstitial bronchopneumonia; otitis media; influenza bacillus meningitis
8 1936	7½ mo.	Lung	Entered hospital because of abdominal tumor; never healthy; poor nutrition; bronchopneumonia, bronchiolitis, capillary bronchitis and otitis media when admitted to hospital 2 weeks before death; died 1 week after removal of cystic abdominal tumor	Interstitial bronchopneumonia; lymphocytic infiltration of salivary gland but no inclusions found
9 1936	14 mo.	Lungs	Ill off and on for 6 weeks with grip and colds; 3 weeks before death admitted to hospital with otitis media, fever and cough; rickets; pneumonia of upper lobe, rt. lung	Interstitial bronchopneumonia with small bronchiectatic abscesses; empyema, bilateral; bilateral focal emphysema; extensive lymphocytic infiltration of salivary gland; S. R. hemolyticus and pneumococcus type IV cultured from empyema fluid

* S. T. S. stands for serologic test for syphilis.

TABLE 1.—Summary of Seventeen of Twenty Cases of Inclusion Disease in Children—Continued

Case and Year	Age of Patient	Organs Showing Inclusions	Clinical History and Findings	Autopsy Observations
10 1945	5 mo.	Lungs; salivary glands not examined	None	Interstitial bronchopneumonia; metaplasia of bronchial epithelium
11 1951	2 mo.	Lung, adrenal, colon (mononuclear cells of mucosa); salivary glands not examined	Cough for 10 days; vomited frequently; difficulty in breathing and cyanosis; no fever or convulsions; heart enlarged and displaced to right; physical signs of bronchopneumonia; WBC 25,000, 64% PMN	Fibrocystic disease of pancreas; purulent bronchitis; interstitial bronchopneumonia; focal necrosis in adrenal; hemosiderin in spleen and liver; Staph. aureus cultured from lungs
12 1964	4 mo.	Duodenum (Brunner's glands), salivary gland	Failed to gain weight properly; repeated spells of vomiting with high fever; 3 weeks before death onset of diarrhea and high fever; 1½ weeks before death wheezing and cough; death 4 days after entering hospital	Fibrocystic disease of pancreas; interstitial pneumonia; small bronchiectatic abscesses containing gram positive cocci; fatty liver
13 1957	3 mo.	Pancreas, adrenal, salivary gland	Vomiting of increasing severity beginning soon after birth; entered hospital 11 days before death; acidosis; fluids poorly absorbed; serum proteins 1.45 A/2.19 G; cyanosis; breathing labored	Fibrocystic disease of pancreas; acute necrotizing tracheitis and bronchitis; bronchopneumonia; fatty metamorphosis of the liver; generalized edema
14 1940	3½ mo.	Kidneys, pancreas, salivary gland	Cough, poor appetite and failure to gain weight for 6 weeks; diarrhea 3 weeks; death 1 week after entering hospital	Fibrocystic disease of pancreas; purulent bronchitis and bronchiolitis; abscess of rt. lung; bronchopneumonia; squamous metaplasia of epithelium of bronchi, ducts of salivary gland and pancreas, and renal pelvis; hemolytic Staph. aureus cultured from lungs
15 1949	3 mo.	Lungs, kidneys, pancreas, duodenum (Brunner's glands), salivary gland	Cough and diarrhea since birth; 3-6 stools daily; failure to gain weight; cough more severe prior to admission to hospital 4 days before death; fever; rales, patchy infiltration of lungs and increased breath sounds	Fibrocystic disease of pancreas; bronchopneumonia; bronchiolitis; squamous metaplasia of tracheal epithelium; fatty metamorphosis of the liver; Pseudomonas aeruginosa cultured from lungs
16 1956	3½ yr.	Lung, liver, adrenal	History of pertussis for 1 mo.; a sibling also had pertussis; convulsions began 3 days before death; signs of pneumonia in both lungs; coma; cyanosis; rapid difficult respirations; high fever; WBC 40,000, 75% lymphocytes	Interstitial bronchopneumonia; focal necrosis in adrenal and liver; small hemorrhages about blood vessels of brain; no changes seen in several series of sections of salivary gland
17 1946	3 mo.	Lungs, kidneys; salivary glands not examined	Edema noted 2 days before admission to hospital; edema increased in hospital; death during transfusion of doubly concentrated plasma 11 days after entry to hospital	Lipoid nephrosis; congestion and edema of lungs; ascites (300 cc.); focal necrosis in liver

was no recognized bacterial infection. Inclusions in the cells of several organs, a cellular reaction associated with the cellular inclusions in the kidneys, slight hepatic damage and acute interstitial pneumonitis were the only pathologic conditions observed at autopsy. In the second case (table 1) the early symptoms were severe cough, vomiting and loss of weight beginning two months before the infant's death. At autopsy there was interstitial pneumonia with cellular inclusions in the lungs in addition to focal acute bronchopneumonia. It is probable that the salivary gland virus was the cause of the interstitial pneumonia and that this produced the early respiratory symptoms. Sulfanilamide was given orally for one week three weeks before the death of the infant. This medication may have been responsible for anemia, thrombocytopenia and increased bleeding tendency which occurred later. However, these symptoms have been manifestations of inclusion disease in other infants. The widespread distribution of the characteristic inclusions indicated that a generalized salivary gland virus infection was present. Inclusions

were observed in large mononuclear cells of the mucosa of the ileum, but there were no clinical symptoms of enteritis. The acute bacterial bronchopneumonia was probably a contributing cause of death.

Four cases (table 1, cases 3 to 6) are summarized in each of which severe diarrhea was the predominant symptom. In these infants the characteristic inclusions were present in large mononuclear cells in the mucosa of the ileum or the colon. In one instance (table 1, case 5) inclusions were observed only in the intestine and the salivary glands. This child was ill for 2½ months, and chronic ulcerative colitis was present at autopsy. The inclusions may have been associated with a secondary viral disease, but, if this was true, the primary cause of the intestinal lesions was not ascertained. There were no gross anatomic lesions in the intestines of the other three infants (table 1, cases 3, 4 and 6). No ulcer or acute inflammation of the mucosa was observed in microscopic sections of the intestines, but in the mucosa in each case there were a moderate number of large mononuclear wandering cells containing the characteristic intranuclear inclusions. One of these three infants was premature and the other two had pneumonia before the onset of the diarrhea; however, none of the children was considered to be in poor general condition before the diarrhea appeared. Viral enteritis may well have been the primary cause of the diarrhea and responsible for the infants' deaths.

Four other infants (table 1, cases 7 to 10) had interstitial bronchopneumonia, and inclusions were found only in cells of the lungs. In one instance (case 10) no history was available and no cause of death other than the interstitial pneumonia was found. The other three infants had symptoms of respiratory disease. One of the three (case 7) had a brassy cough for three weeks. Severe diarrhea was also present in this child, but there were no gross lesions in the intestinal tract, and sections of the intestines were not available for study. The immediate cause of death in this instance was acute meningitis caused by the influenza bacillus. Another infant (case 8), 7½ months of age, was poorly nourished and had never been healthy. Diagnoses of bronchopneumonia, bronchiolitis and otitis media were made when the infant entered the hospital two weeks before death. Interstitial pneumonia, associated with the presence of inclusions in cells of the lungs, was present at autopsy and may have contributed to death, which occurred one week after a cystic abdominal tumor had been removed from the region of the tail of the pancreas. The fourth child in this group (table 1, case 9) had respiratory infections for six weeks before his death at 14 months of age. Interstitial pneumonia was present. There were also small bronchiectatic abscesses in the upper lobe of the right lung and bilateral empyema. Hemolytic streptococci and pneumococci type IV were cultured from the empyema fluid. The bacterial infection was probably the immediate cause of death. From the data available it is impossible to decide whether the

viral infection preceded or followed the bacterial infection or what part it played in the child's fatal illness.

In the remaining seven cases presented in table 1 (cases 11 to 17) the inclusion disease was regarded as a secondary viral infection occurring during the course of another disease. In five instances the principal disease was fibrocystic disease of the pancreas. Bacterial infection of the lungs was present in each case. In two the pneumonia was of the interstitial type, and inclusions were seen in the lungs in one of these. The only other tissue reaction associated with the inclusions was focal necrosis of the adrenals in one instance. The metabolic disorder resulting from pancreatic insufficiency may have been of importance in facilitating the dissemination of the salivary gland virus. In those instances of the association of fibrocystic disease of the pancreas and inclusion disease which we have observed in recent years, the infants entered St. Louis Children's Hospital only a short time before death, and correction of the nutritional deficiencies had not been instituted prior to admission to the hospital. Inclusion disease cannot have been more than a minor contributing factor in the death of these children.

The oldest child (table 1, case 16) of the present series was 3½ years of age. She had pertussis for one month before death. She had been exposed to pertussis, and there was no reason to doubt the correctness of this diagnosis. The principal pathologic finding was interstitial pneumonia. Inclusions were present in the lungs (fig. 6) and, associated with small foci of necrosis, in the liver and the adrenals. In this instance, also, dissemination of the salivary gland virus may have occurred during the course of the primary disease, and may be considered no more than a contributory cause of death.

The last case of inclusion disease recorded in table 1 (case 17) was that of an infant who had lipoid nephrosis (a nephrotic syndrome due to an undetermined cause). The characteristic inclusions in cells of the lungs and kidneys were the only anatomic changes attributable to a virus. The viral infection was apparently incidental and bore no causal relation to the disease for which the infant was being treated.

ANALYSIS OF REPORTED CASES AND COMPARISON WITH THOSE IN THE PRESENT SERIES

A summary of the 69 reported cases in which intracellular inclusions characteristic of generalized salivary gland virus infection occurred in the viscera of infants and young children is given in table 2. In at least 16 of the 69 cases the prominent manifestations of the disease associated with the inclusions were those of a blood dyscrasia or of hepatic damage like those observed in our first three cases. Both jaundice and petechiae or purpura occurred in nine instances (table 2, cases 4, 10, 31, 51, 52, 58, 59, 63 and 69). In seven of these nine extramedullary hemopoiesis was observed, and in five some anatomic change other than extra-

medullary hemopoiesis was present in the liver. The lesions described in the livers included periportal infiltration, bile stasis, cytolytic necrosis and in two instances biliary cirrhosis. In another case (68) hematemesis, melena, anemia and jaundice occurred, and diffuse hepatitis with fibrosis was present at autopsy. Purpura or a hemorrhagic diathesis without jaundice was present in 4 cases (table 2, cases 10, 52, 62 and 67). In one of the four anemia and marked extramedullary hemopoiesis occurred; in another splenomegaly was recorded and in a third anemia and thrombocytopenia. In two instances (table 2, cases 18 and 24), in which neither jaundice nor hemorrhages were recorded, diagnoses of erythroblastosis and of erythroleukoblastosis were made.

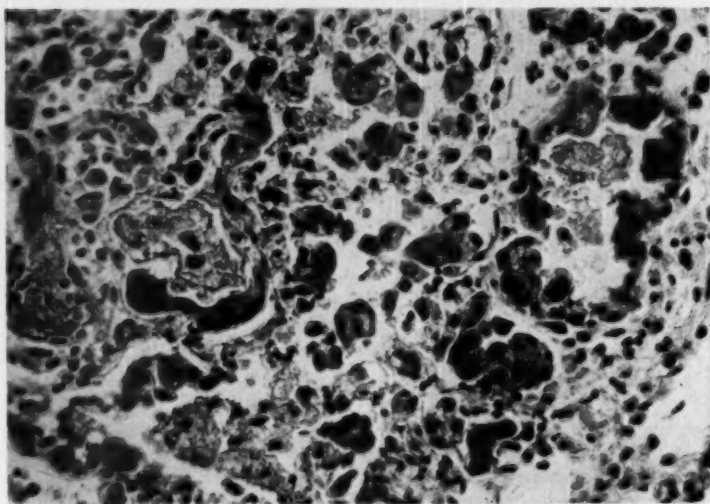


Fig. 6 (case 16).—Lung with interstitial pneumonia. Hypertrophied cells containing intranuclear inclusions line alveoli and lie free in the alveolar lumens. The cytoplasmic inclusions are barely visible.

Only one of the 16 infants having a blood dyscrasia or a hepatic disease was over 2 months of age at the time of death, and this infant had thrombocytopenia, purpura, anemia and enlargement of spleen and liver during the neonatal period. Thus, in newborn infants and infants a few weeks of age inclusion disease has been manifested in many instances by a blood disorder, hepatic damage or both. Two of the instances of inclusion disease of this type, occurring in newborn infants, in which the predominating manifestations were regarded as hemolytic anemia, were reported by Cappell and McFarlane.⁴ They also pointed out that other reported cases of inclusion disease in young infants bore

a resemblance to those which they reported and stated that this disease was probably confused with congenital syphilis in the older literature and more recently with erythroblastosis fetalis and hemorrhagic disease of the newborn.

It is obvious from the widespread distribution of inclusions in some instances that the salivary gland virus may affect many organs of the body. Therefore, it is to be expected that the tissues most damaged are not the same in every instance. Inclusions in cells associated with focal interstitial inflammation of the kidney have been described frequently, but in only a few instances has the renal damage been severe. In one case reported by Wyatt and associates (table 2, case 67) oliguria was present, and an anatomic diagnosis of severe nephrosis was made. A diagnosis of exudative nephritis was made in another instance (table 2, case 13). In the kidneys of each of the three infants in our series who died in the neonatal period, there were many cells containing inclusions and there was associated focal cellular infiltration. In one instance there was dilatation of the collecting tubules with casts consisting in part of fragments of desquamated tubular epithelium.

Involvement of the brain also occurs. In two of the cases reported by Wyatt and associates (table 2, cases 67 and 68) and in one of our series there was ependymitis. Inclusions were present in the meninges in another of our cases, but there was little inflammatory reaction. Worth and Howard reported hemorrhage and necrosis of the basal ganglions (table 2, case 63). Inclusions were present in ganglion and glial cells. Necrosis, mononuclear infiltration and intranuclear inclusions were reported in the brain by Kinney.

Viral enteritis has been another manifestation of inclusion disease. Inclusions in the cells of the intestines of infants have been reported only twice previously (table 2, cases 63 and 67). Diarrhea occurred in both instances. In our series inclusions were present in the intestinal tract in nine cases. In three they were found only in a small number of the epithelial cells of duodenal mucosa. In five others intranuclear inclusions were present in large mononuclear cells of the mucosa of the ileum, and in one, in large mononuclear cells in granulation tissue at the base of ulcers of the colon. The predominant clinical symptom was diarrhea in four of the infants whose intestines showed these lesions in the ileum or the colon.

Interstitial or chronic pneumonia has been reported frequently and appears to be one of the commonest manifestations of the disease in infants beyond the neonatal period. Twenty-eight instances (table 2, cases 15, 19 to 23, 33 to 50, 54 to 57, and 66) of inclusion disease associated with pertussis have been reported; 18 of these, by McCordock and Smith.²⁶ Interstitial or chronic pneumonia was described in most of these cases. The association of inclusion disease and pertussis is

TABLE 2.—Summary of Sixty-Nine Reported Cases of Inclusion Disease in Infants and Children

Case*	Age	Clinical and Autopsy Data	Organs with Inclusions
1 ^a	Stillborn	History of syphilis in father; lesions with inclusions regarded as syphilitic	Kidneys, lungs, liver
2 ^a	Newborn	Congenital syphilis	Kidneys
3 ^a	Stillborn	Congenital syphilis	Kidneys, lungs, liver
4 ^b	8 days	Jaundice; purpura; fibrosis and mononuclear infiltration in liver, bile stasis; regarded as infection of liver	Liver
5 ^c	16 days	Purpura; splenomegaly; no evidence of syphilis	Thyroid
6 ^c	Stillborn	Cellular infiltration in liver and kidneys; no evidence of syphilis	Kidneys, lungs, liver
7 ^a	2 mo.	Family history of syphilis negative; chronic interstitial pancreatitis; organizing pneumonia; ulcerative and scaly skin lesions regarded as syphilitic	Lungs
8 ^a	6 weeks	No evidence of syphilis, no spirochetes; pancreatic fibrosis and dilation of ducts; subacute pneumonia	Kidneys, lungs, liver
9 ^c	15 mo.	Diphtheria	Kidneys
10 ^a	1½ mo.	Family history of syphilis negative; no spirochetes; jaundice; petechiae; splenomegaly; cirrhosis with bile stasis; cellular infiltration in kidney	Kidneys, lungs
11 ^b	Stillborn	Premature; cellular infiltration in kidney	Kidneys
12 ^a	8 weeks	Hydrocephalus; cellular infiltration in kidney	Kidneys
13 ^b	2 mo.	Jaundice; slight induration of liver and spleen; colitis; diffuse exudative nephritis; scaly lesions of palms and soles regarded as syphilitic	Kidneys
14 ^c	Newborn	Premature; hypoplasia and stenosis of ileum and colon; hydrops; slight interstitial fibrosis of pancreas; no spirochetes; mother's S. T. S. negative	Kidneys, lungs, liver, thyroid, pancreas
15 ^b	3½ mo.	Pertussis	Lungs
16 ^a	15 days	Premature; staphylococcal pyemia; hemorrhagic diathesis; no spirochetes; mother's S. T. S. negative	Kidneys, lungs, liver, pancreas, thyroid, salivary glands, epididymis
17 ^b	30 days	Hemorrhagic diathesis	Kidneys, lungs, liver, pancreas, thyroid
18 ^a	2 days	Erythroblastosis	Kidneys, lungs, liver, pancreas
19-22 ^b	3½ mo. to 2 yr.	Pertussis	Lungs
24 ^a	6 hr.	Erythroleukoblastosis; ascites; hemopoiesis in many organs; enlarged liver and spleen; periportal infiltration; subarachnoidal hemorrhage	Kidneys, liver
25 ^a	4 mo.	Chronic pneumonia; peribronchial infiltration; pancreatic fibrosis and dilation of ducts	Lungs
26 ^a	3 mo.	Chronic pneumonia; peribronchial infiltration; pancreatic fibrosis and dilatation of ducts; focal necrosis in liver and adrenals	Kidneys, lungs, liver, salivary gland
27 ^a	4½ mo.	Malnutrition; rickets; xerophthalmia; convulsions; chronic pneumonia; peribronchial infiltration; focal infiltration in kidneys; no spirochetes	Lungs
28 ^a	5 mo.	Chronic pneumonia; peribronchial infiltration; lung abscesses	Lungs
29 ^a	2 yr.	Chronic pneumonia; peribronchial infiltration; gangrene of cheeks, palate and gums; ulcerative colitis	Lungs
30 ^a	3 mo.	Chronic pneumonia; peribronchial infiltration; cleft palate	Lungs
31 ^a	1 day	Jaundice; multiple hemorrhages; hemopoiesis in liver and spleen; focal infiltration in kidneys; slight pancreatic fibrosis; no spirochetes	Lungs
32 ^a	6 mo.	Unexplained cerebral hemorrhage; no spirochetes	Liver
33-50 ^a	3 mo. to 5 yr.; 13 yr. (one case)	Pertussis	Lungs in all cases, adrenal and liver in some cases

TABLE 2—Summary of Sixty-Nine Reported Cases of Inclusion Disease in Infants and Children—Continued

Case*	Age	Clinical and Autopsy Data	Organs with Inclusions
51 ^a	1 day	Jaundice; ecchymosis; bile stasis in liver; enlarged spleen; marred hemopoiesis in liver; mother's S. T. S. negative	Kidneys, lungs, liver
52 ^a	1 day	Premature; erythromyelosis; jaundice; purpura; anemia; cellular infiltration in kidney	Kidneys, lungs, liver, thyroid
53 ^a	14 days	Diarrhea; jaundice; necrosis and mononuclear infiltration in brain	Kidneys, lungs, liver, pancreas, brain
54-57 ^a	Infants	Pertussis	Lungs
58 ^a	1 day	Premature; hemolytic disease; jaundice; petechiae; erythroblastemia; splenomegaly; S. T. S. negative; no evidence of maternal isoimmunization; extramedullary hemopoiesis; cytolytic necrosis and bile stasis in liver; focal lymphocytic infiltration in kidneys	Kidneys, lungs, liver, pancreas
59 ^a	16 hr.	Premature; jaundice; purpura; erythroblastosis; no evidence of maternal isoimmunization	Kidneys, lungs, liver
60 ^a	2½ mo.	Conjunctivitis; inflammation of orbital tissue; enlarged submaxillary glands; fibrosis of thyroid; interstitial pneumonia; fibrosis and focal infiltration of liver; areas of necrosis in adrenal; S. T. S. negative	Kidneys, lungs, liver, pancreas, thyroid, salivary gland, eyeball, spleen
61 ^a	6 days	Maternal history of syphilis; sepsis; leukemia; hemorrhagic pneumonia	Kidneys, liver, pancreas, thyroid, salivary glands
62 ^a	Newborn	Prematurity; anemia; petechiae; hemopoiesis in all organs; phlegmon of thigh; bronchopneumonia	Kidneys, lungs, liver, pancreas, thyroid, salivary glands
63 ^a	6 weeks	Hemorrhage from umbilical stump in neonatal period; diarrhea; anemia; jaundice; petechiae; excoriations of buttocks; enlarged spleen and liver; extramedullary hemopoiesis in liver; hemorrhage and necrosis of basal ganglia; S. T. S. negative	Kidneys, lungs, liver, pancreas, thyroid, adrenal, pituitary, stomach, intestine, brain, testes, epididymis, heart, spleen, skin, bone marrow
64 ^a	Stillborn	Full term infant; focal interstitial pneumonitis; marked hemopoiesis in liver and spleen	Kidneys, lungs, adrenals
65 ^a	14 mo.	Interstitial pneumonitis; mononuclear infiltration of liver; maculopapular skin rash	Kidneys, lungs
66 ^a	14 mo.	Pertussis; interstitial pneumonitis; mononuclear infiltration of liver	Lungs, liver
67 ^a	13 weeks	Anemia; thrombocytopenia; purpura; enlarged spleen and liver in neonatal period; S. T. S. negative; no evidence of maternal isoimmunization; interstitial pneumonitis; mononuclear infiltration in kidneys; severe nephrosis; ileitis; ependymitis	Kidneys, lungs, liver, pancreas, adrenals, brain, ileum
68 ^a	7 weeks	Diarrhea; hematemesis; melena; anemia; jaundice; edema; ascites; hematuria; S. T. S. negative; no evidence of maternal isoimmunization; diffuse hepatitis and fibrosis; interstitial pneumonitis; ependymitis	Kidneys, lungs, liver, pancreas, brain
69 ^a	5 weeks	Purpura; hepatosplenomegaly; jaundice; diarrhea; S. T. S. negative in mother and infant; blood of mother Rh negative, that of infant Rh positive; biliary cirrhosis; extramedullary hemopoiesis	Kidneys, lungs, liver, pancreas, thyroid, parathyroid, salivary gland

* Superior numbers indicate footnotes related to the text. Superior letters indicate the footnotes appended to this table.

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- (b) Mouchet, R.: *Arch. de méd. expér. et d'anat. path.* **23**: 115, 1911.
- (c) Pettavel, C. A.: *Virchows Arch. f. path. Anat.* **306**: 1, 1911.
- (d) Smith, A. J., and Weidman, F. D.: *Univ. Pennsylvania M. Bull.* **33**: 285, 1910-1911.
- (e) Smith, A. J., and Weidman, F. D.: *Am. J. Trop. Dis.* **3**: 256, 1914.
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difficult to interpret. It is possible that clinical symptoms due to inclusion disease have been mistaken for those of pertussis in some instances, but in most instances the diagnosis of pertussis appears to have been unquestioned. Pertussis was diagnosed in 1 case of the present series.

Intracellular inclusions have occurred in the pancreas in 13 of the cases in the literature and in 8 of our series. Interstitial fibrosis, usually slight in amount, or cellular infiltrations have been present, but no symptoms of pancreatic disease which could be attributed to the viral infection. The occurrence of inclusion disease and fibrocystic disease of the pancreas has been noted in five cases in this series. Two of the cases reported by McCordock and Smith (table 2, cases 25 and 26), in which fibrosis of the pancreas and dilatation of the pancreatic ducts were recorded, have been reviewed. These are also examples of fibrocystic disease of the pancreas. In addition, the description of the pancreas in another report, that of Goodpasture and Talbot (table 2, case 8), may be interpreted as a description of fibrocystic disease. Interstitial or chronic pneumonia and pulmonary cells containing inclusions have been observed in some of the infants having fibrocystic disease of the pancreas.

INCIDENCE OF INCLUSION DISEASE

None of the cases of inclusion disease cited in this report concerned stillborn infants; however, five stillborn infants having this disease have been observed by others. Approximately three fourths of the 89 cases of inclusion disease, including those in this report, have involved infants 2 years of age or younger. Only one child was over 5 years of age. Twenty-one infants died within the neonatal period of one month; nine died on the first day of life and eight others within the first two weeks. Fifteen of the 16 cases which we observed from 1934 to 1949, inclusive, were encountered in 1,411 autopsies of infants and children from St. Louis Children's Hospital, an incidence of slightly over 1 per cent. Wyatt and associates⁸ noted an incidence of 1.1 per cent in autopsies of 461 infants and children from other St. Louis hospitals. Only three cases of inclusion disease of adults have been reported.²⁰ In addition the finding of inclusions in isolated lesions in six adults has been reported.²¹ The isolated lesions included ulcers of the esophagus or the

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stomach and, in one instance, a localized granuloma of the anus. Instances of the disease in infants and children have been reported from many parts of the world. The fact that 52 of a total of 89 cases in children, including those in this report, have been observed in St. Louis may be due only to the special interest of the pathologists.

POSSIBLE FACTORS PREDISPOSING TO GENERALIZED INFECTION

It appears certain that salivary gland virus infection may occur during intrauterine life, since inclusion disease has been observed in stillborn and newborn infants. In other instances, infants dying in the first days or weeks of life have had symptoms from birth or shortly thereafter. By what mechanism the fetal infection occurs is unknown; also, it is unknown how long a latent infection may persist in the salivary gland, or under what conditions dissemination of the virus may occur. There has been no apparent infection in the mothers of stillborn or newborn infants having inclusion disease. One may speculate as to whether the mother had a recent inapparent infection of the salivary gland during pregnancy, with the virus being disseminated in the blood stream and invading the fetal tissue, or had a recrudescence of a latent infection present since early life. It may be significant that the mothers of many of the infants have been young. Perhaps a first infection, delayed until early adult life, occurred during pregnancy and the virus invaded the blood stream. Evidence obtained from experiments on guinea pigs indicates that fetal tissues are more susceptible to virus infection than are adult tissues.¹⁵ The increased susceptibility of fetal tissues may account for the fact that this disease may appear in the infant *in utero* despite the fact that the mother shows no clinical evidence of the disease.

The factors which predispose to the generalized disease in older infants and children and adults have not been established. There is suggestive evidence that disturbances of metabolism, such as those related to vitamin deficiencies, may be involved. The observation that the generalized disease occurred in a number of infants having fibrocystic disease of the pancreas would be consistent with this hypothesis. Further evidence is given by two of the three cases reported in adults in which inclusions of the salivary gland virus type occurred in the viscera. One of these adults was a 63 year old woman who had a deficiency in the vitamin A content of the blood and lesions of the skin suggestive of a nutritional disease.^{20b} Severe interstitial pneumonia apparently due to the salivary gland virus was the cause of death. Another adult had leukemia and received 4-aminopteroylaspartic acid (amino-an-fol*) over a period of 5½ months.^{20c} Focal interstitial pneumonia associated with cells in the lungs containing the characteristic intranuclear inclusions was present. The viral pneumonia was believed not to be an important factor in the patient's death. Since these inclusions are found rarely in the tissues of adults, their occurrence in this man is intriguing. He was

49 years of age and in amino-an-fol* had received a substance which is known to affect cellular metabolism. Another observation, interesting in this respect, is that a spontaneous disease which we are interpreting as a generalized salivary gland virus infection occurred in several guinea pigs which had received 4-amino-pteroylglutamic acid (aminopterin*) over a period of approximately three weeks. Investigation of the effects of metabolic disturbances in causing dissemination of the salivary gland virus in rodents is indicated.

SUMMARY AND CONCLUSIONS

The inclusion-bearing cells observed in inclusion disease have a distinctive morphologic appearance and are pathognomonic of the disease. The presence of intracellular inclusions comparable to those associated with known viral diseases and the analogy noted between the natural and experimental salivary gland virus disease of rodents and the human inclusion disease offers reasonable evidence that this disease is caused by a virus and that the virus concerned is the salivary gland virus.

Twenty cases of inclusion disease of infants and children are presented. In three instances the disease was observed in infants dying in the neonatal period.

The following conclusions are derived from our observations and those reported in the literature:

Generalized salivary gland virus infections occur, in the majority of instances, during the first two years of life. The infection may occur *in utero* without the mother showing any evidence of the infection. Death due to the intrauterine infection may occur *in utero* or in the neonatal period.

In infants under 2 months of age generalized salivary gland virus infection is usually a primary fatal disease, and its most frequent clinical and anatomical manifestations are those of a blood dyscrasia or of hepatic damage. However the character of the disease may vary, since the viral infection may involve, with variable intensity, many organs of the body, including especially the liver, the lungs, the kidneys, the brain and the intestines.

In children over 2 months of age inclusion disease is associated with diverse clinical histories and anatomic changes. At times it may be the principle disease; more often it is associated with another primary disease and plays only a minor role in the child's illness.

There is suggestive evidence that disturbances of cellular metabolism such as those occurring in vitamin deficiencies may predispose to generalized salivary gland virus infections.

The association of pertussis and generalized salivary gland virus infection in approximately one third of the cases, including those in the present report, is noted but remains unexplained.

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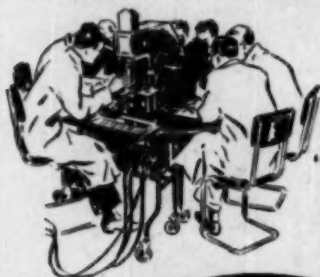
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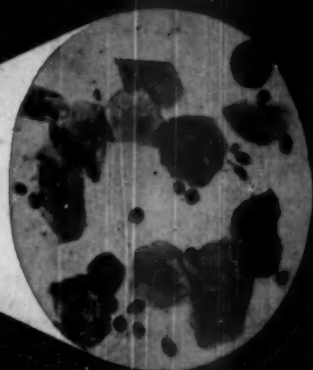


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The 64,000 lumen intensity of the Scopicon high pressure mercury lamp permits auditorium demonstration of vaginal, uterine, bronchial and gastric smears and smears made from urine or other body exudates, even under oil-immersed microscope objectives. In projected images six feet or more across, every detail of the normal and abnormal cells can be seen due to the pinpoint character of the 1 mm. square (approx.) focal spot employed. The Scopicon light is steady, flickerless, its brilliant white color exhibiting biological stains to the greatest advantage. May we send you the brochure describing this versatile instrument?

**microprojection
photomicrography**